



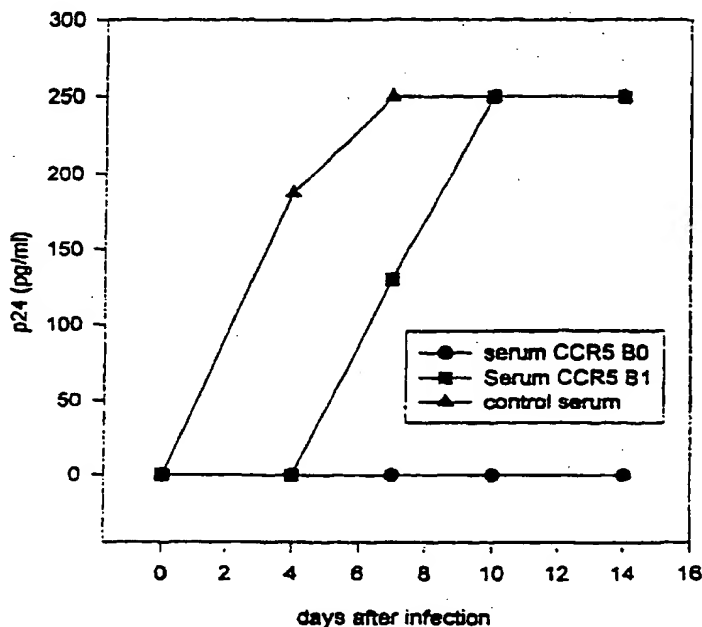
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: C-C CKR-5, CC-CHEMOKINES RECEPTOR, DERIVATIVES THEREOF AND THEIR USES

## (57) Abstract

The present invention is related to new peptides and the nucleic acid molecules encoding said peptides. The present invention concerns also the vector comprising said nucleic acid molecules, cells transformed by said vector, inhibitors directed against said peptides or said nucleic acid molecules, a pharmaceutical composition and a diagnostic and/or dosage device comprising said products, and non human transgenic animals expressing the peptides according to the invention or the nucleic acid molecules encoding said peptides.



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**CC CCR-5, CC-CHEMOKINES RECEPTOR, DERIVATIVES THEREOF AND THEIR USES**

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Field of the present invention.

The present invention concerns new peptides and the nucleic acid molecules encoding said peptides, the  
15 vector comprising said nucleic acid molecules, the cells transformed by said vector, inhibitors directed against said peptides or said nucleic acid molecules, a pharmaceutical composition and a diagnostic and/or dosage device comprising said products, and non human transgenic  
20 animals expressing the peptides according to the invention or the nucleic acid molecules encoding said peptides.

The invention further provides a method for determining ligand binding, detecting expression, screening for drugs binding specifically to said peptides and  
25 treatments involving the peptides or the nucleic acid molecules according to the invention.

Technological background and state of the art.

Chemotactic cytokines, or chemokines, are  
30 small signalling proteins that can be divided in two subfamilies (CC- and CXC-chemokines) depending on the relative position of the first two conserved cysteines.

Interleukin 8 (IL-8) is the most studied of these proteins, but a large number of chemokines (Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), Monocyte Chemoattractant Protein 1 (MCP-1), Monocyte Chemoattractant Protein 2 (MCP-2), Monocyte Chemoattractant Protein 3 (MCP-3), Growth-Related gene product  $\alpha$  (GRO $\alpha$ ), Growth-Related gene product  $\beta$  (GRO $\beta$ ), Growth-Related gene product  $\gamma$  (GRO $\gamma$ ), Macrophage Inflammatory Protein 1  $\alpha$  (MIP-1 $\alpha$ ) and  $\beta$ , etc.) has now been described [4]. Chemokines play fundamental roles in the physiology of acute and chronic inflammatory processes as well as in the pathological dysregulations of these processes, by attracting and simulating specific subsets of leucocytes [32]. RANTES for example is a chemoattractant for monocytes, memory T-cells and eosinophils, and induces the release of histamine by basophils. MCP-1, released by smooth muscle cells in arteriosclerotic lesions, is considered as the factor (or one of the factors) responsible for macrophage attraction and, therefore, for the progressive aggravation of the lesions [4].

MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES chemokines have recently been described as major HIV-suppressive factors produced by CD8<sup>+</sup> T-cells [9]. CC-chemokines are also involved in the regulation of human myeloid progenitor cell proliferation [6, 7].

Recent studies have demonstrated that the actions of CC- and CXC-chemokines are mediated by subfamilies of G protein-coupled receptors. To date, despite the numerous functions attributed to chemokines and the increasing number of biologically active ligands, only six functional receptors have been identified in human. Two receptors for interleukin-8 (IL-8) have been described [20,



29]. One (IL-8RA) binds IL-8 specifically, while the other (IL-8RB) binds IL-8 and other CXC-chemokines, like GRO. Among receptors binding CC-chemokines, a receptor, designated CC-chemokine receptor 1 (CCR1), binds both  
5 RANTES and MIP-1 $\alpha$  [31], and the CC-chemokine receptor 2 (CCR2) binds MCP-1 and MCP-3 [8, 44, 15]. Two additional CC-chemokine receptors were cloned recently: the CC-chemokine receptor 3 (CCR3) was found to be activated by  
10 RANTES, MIP-1 $\alpha$  and MIP-1 $\beta$  [10]; the CC-chemokine receptor 4 (CCR4) responds to MIP-1, RANTES and MCP-1 [37]. In addition to these six functional receptors, a number of orphan receptors have been cloned from human and other species, that are structurally related to either CC- or CXC-chemokine receptors. These include the human BLR1 [13],  
15 EBI1 [5], LCR1 [21], the mouse MIP-1 RL1 and MIP-1 RL2 [17] and the bovine PPR1 [25]. Their respective ligand(s) and function(s) are unknown at present.

#### Summary of the invention

20 The present invention is related to a peptide having at least an amino acid sequence which presents more than 80%, advantageously more than 90%, preferably more than 95%, homology with the amino acid sequence as represented in SEQ ID NO. 1.

25 Preferably, said peptide has also at least an amino acid sequence which presents more than 80%, advantageously more than 90%, preferably more than 95%, homology with the amino acid sequence as represented in SEQ ID NO. 2.

30 According to another embodiment of the present invention, the peptide has at least an amino acid sequence which presents more than 80%, advantageously more

than 90%, preferably more than 95%, homology with the amino acid sequence as represented in SEQ ID NO. 3.

The present invention is also related to the amino acid sequence of SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or a portion thereof (represented in the fig. 1).

A "portion of an amino acid sequence" means one or more amino acid segments having the same or improved binding properties of the whole peptide according to the invention. Said portion could be an epitope which is specifically binded by a ligand of the peptide which could be a known "natural ligand" of said peptide, an agonist or an analog of said ligand, or an inhibitor capable of competitively inhibiting the binding of said ligand to the peptide (including the antagonists of said ligand to the peptide).

Specific examples of said portions of amino acid sequence and their preparation process are described in the publication of Rucker J. et al. (Cell, Vol. 87, pp. 437-446 (1996)) incorporated herein by reference.

According to the invention, said portion of the amino acid sequence of the peptide according to the invention comprises the N-terminus segment and the first extracellular loop of the peptide.

Therefore, according to the invention, the amino acid sequence as represented in SEQ ID NO. 1 is the common amino acid sequence of SEQ ID NO. 2 and of SEQ ID NO. 3 (see also figure 1). Therefore, a first industrial application of said amino acid sequence is the identification of the homology between said amino acid sequence and the screening of various mutants encoding a different amino acid sequence than the one previously described, and the identification of various types of

patient which may present a predisposition or a resistance to the disorders described in the following specification.

Preferably, the peptide according to the invention or a portion thereof is an active CC-chemokine  
5 receptor.

Advantageously, the CC-chemokine receptor according to the invention is stimulated by the MIP-1 $\beta$  chemokine at a concentration less or equal to 10 nm, and is advantageously also stimulated by the MIP-1 $\alpha$  or RANTES  
10 chemokines. However, said chemokine receptor is not stimulated by the MCP-1, MCP-2, MCP-3, IL-8 and GRO $\alpha$  chemokines.

In addition, the peptide according to the invention or a portion thereof is also a receptor of HIV  
15 viruses or a portion of said HIV viruses.

It is meant by "HIV viruses", HIV-1 or HIV-2 and all the various strains of HIV viruses which are involved in the development of AIDS. It is meant by a "a portion of HIV viruses", any epitope of said viruses which  
20 is able to interact specifically with said receptor. Among said portions of viruses which may be involved in the interaction with the peptide according to the invention, are peptides encoded by the ENV and GAG viruses genes.

Preferably, said portion of HIV viruses is  
25 the glycopeptide gp120/160 (membrane-bound gp160 or the free gp derived therefrom) or a portion thereof.

It is meant by a "portion of the glycopeptide gp120/160" any epitope, preferably an immuno-dominant epitope, of said glycopeptide which may interact  
30 specifically with the peptide according to the invention, such as for instance the V3 loop (third hypervariable domain).



According to another embodiment of the present invention, the peptide according to the invention is an inactive CC-chemokine receptor. An example of such inactive CC-chemokine receptor is encoded by the amino acid sequence as represented in SEQ ID NO. 2.

It is meant by an "inactive CC-chemokine receptor" a receptor which is not stimulated by any known CC-chemokine, especially the MIP-1 $\beta$ , MIP-1 $\alpha$  or RANTES chemokines.

The peptide represented in SEQ ID NO. 3 according to the invention is an inactive receptor which is not a receptor of HIV viruses or of a portion of said HIV viruses, which means that said inactive receptor does not allow the entry of said HIV viruses into a cell which presents at its surface said inactive receptor.

Advantageously, the peptide according to the invention is a human receptor.

The present invention concerns also the nucleic acid molecule having more than 80%, preferably more than 90%, homology with one of the nucleic acid sequences of SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3 shown in the figures 1.

Preferably, said nucleic acid molecule has at least the nucleic acid sequence shown in SEQ ID NO. 1, SEQ ID NO. 2 or SEQ ID NO. 3 of figure 1 or a portion thereof.

It is meant by a "portion of said nucleic acid molecule" any nucleic acid sequence of more than 15 nucleotides which could be used in order to detect and/or reconstitute said nucleic acid molecule or its complementary strand. Such portion could be a probe or a primer which could be used in genetic amplification using the PCR, LCR, NASBA or CPR techniques for instance.

The present invention concerns more specifically the nucleic acid molecules encoding the peptide according to the invention. Said nucleic acid molecules are RNA or DNA molecules such as a cDNA molecule  
5 or a genomic DNA molecule.

The present invention is also related to a vector comprising the nucleic acid molecule according to the invention. Preferably, said vector is adapted for expression in a cell and comprises the regulatory elements  
10 necessary for expressing the amino acid molecule in said cell operatively linked to the nucleic acid sequence according to the invention as to permit expression thereof.

Preferably, said cell is chosen among the group consisting of bacterial cells, yeast cells, insect  
15 cells or mammalian cells. The vector according to the invention is a plasmid, preferably a pcDNA3 plasmid, or a virus, preferably a baculovirus, an adenovirus or a semliki forest virus.

The present invention concerns also the cell,  
20 preferably a mammalian cell, such as a CHO-K1 or a HEK293 cell, transformed by the vector according to the invention. Advantageously, said cell is non neuronal in origin and is chosen among the group consisting of CHO-K1, HEK293, BHK21, COS-7 cells.

25 The present invention also concerns the cell (preferably a mammalian cell such as a CHO-K1 cell) transformed by the vector according to the invention and by another vector encoding a protein enhancing the functional response in said cell. Advantageously, said protein is the  
30 Ga15 or Ga16 (G protein,  $\alpha$  subunit). Advantageously, said cell is the cell CHO-K1-pEFIN hCCRS-1/16.

The present invention is also related to a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridising with a unique sequence included within the sequence of the  
5 nucleic acid molecule according to the invention. Said nucleic acid probe may be a DNA or a RNA.

The invention concerns also an antisense oligonucleotide having a sequence capable of specifically hybridising to an mRNA molecule encoding the peptide  
10 according to the invention so as to prevent translation of said mRNA molecule or an antisense oligonucleotide having a sequence capable of specifically hybridising to the cDNA molecule encoding the peptide according to the invention.

Said antisense oligonucleotide may comprise  
15 chemical analogs of nucleotide or substances which inactivate mRNA, or be included in an RNA molecule endowed with ribozyme activity.

Another aspect of the present invention concerns a ligand or an anti-ligand (preferably an  
20 antibody) other than known "natural ligands", which are chosen among the group consisting of the MIP-1 $\beta$ , MIP-1 $\alpha$  or RANTES chemokines, HIV viruses or a portion of said HIV viruses, wherein said ligand is capable of binding to the receptor according to the invention and wherein said anti-  
25 ligand is capable of (preferably competitively) inhibiting the binding of said known "natural ligand" or the ligand according to the invention to the peptide according to the invention.

The exclusion in the above identified  
30 definition of known chemokines, HIV viruses or a portion of said HIV viruses, does not include variants of said "natural" viruses or said "natural" portion which may be

obtained for instance by genetic engineering and which may mimic the interaction of said viruses and portion of said viruses to the peptide according to the invention.

Advantageously, said antibody is a monoclonal  
5 antibody which is preferably directed to an epitope of the peptide according to the invention and present on the surface of a cell expressing said peptide.

Preferably, said antibody is produced by the hybridoma cell AchCCR5-SAB1A7.

10 The invention concerns also the pharmaceutical composition comprising either an effective amount of the peptide according to the invention (in order to delude the HIV virus from the natural peptide present at the surface of a mammalian cell and stop the infection of  
15 said mammalian cell by the HIV virus), or an effective amount of the above identified described ligand and/or anti-ligand, or an effective amount of oligonucleotide according to the invention, effective to decrease the activity of said peptide by passing through a cell membrane  
20 and binding specifically with mRNA encoding the peptide according to the invention in the cell so as to prevent its translation. The pharmaceutical composition comprises also a pharmaceutically acceptable carrier, preferably capable of passing through said cell membrane.

25 Preferably, in said pharmaceutical composition, the oligonucleotide is coupled to a substance, such as a ribozyme, which inactivates mRNA encoding the peptide according to the invention.

Preferably, the pharmaceutically acceptable  
30 carrier comprises a structure which binds to a receptor on a cell capable of being taken up by cell after binding to the structure. The structure of the pharmaceutically

acceptable carrier in said pharmaceutical composition is capable of binding to a receptor which is specific for a selected cell type.

The present invention concerns also a  
5 transgenic non human mammal overexpressing (or expressing ectopically) the nucleic acid molecule encoding the peptide according to the invention.

The present invention also concerns a  
transgenic non human mammal comprising an homologous  
10 recombination knockout of the native peptide according to the invention.

According to a preferred embodiment of the  
invention, the transgenic non human mammal whose genome  
comprises antisense nucleic acid complementary to the  
15 nucleic acid according to the invention is so placed as to be transcribed into antisense mRNA which is complementary to the mRNA encoding the peptide according to the invention and which hybridises to mRNA encoding said peptide, thereby reducing its translation. Preferably, the transgenic non  
20 human mammal according to the invention comprises a nucleic acid molecule encoding the peptide according to the invention and comprises additionally an inducible promoter or a tissue specific regulatory element.

Preferably, the transgenic non human mammal  
25 is a mouse.

The invention relates to a method for  
determining whether a ligand can be specifically bound to  
the peptide according to the invention, which comprises  
contacting a cell transfected with a vector expressing the  
30 nucleic acid molecule encoding said peptide with the ligand under conditions permitting binding of ligand to such peptide and detecting the presence of any such ligand bound

specifically to said peptide, thereby determining whether the ligand binds specifically to said peptide.

The invention relates to a method for determining whether a ligand can specifically bind to a peptide according to the invention, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the ligand with the membrane fraction under conditions permitting binding of the ligand to such peptide and detecting the presence of any ligand bound to said peptide, thereby determining whether the compound is capable of specifically binding to said peptide. Preferably, said method is used when the ligand is not previously known.

The invention relates to a method for determining whether a ligand is an agonist of the peptide according to the invention, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting the activation of a functional peptide response from the cell and detecting by means of a bio-assay, such as a modification in a second messenger concentration (preferably calcium ions or inositol phosphates such as  $IP_3$ ) or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), an increase in the peptide activity, thereby determining whether the ligand is a peptide agonist.

The invention relates to a method for determining whether a ligand is an agonist of the peptide according to the invention, which comprises preparing a

cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under  
5 conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a modification in the production of a second messenger (preferably inositol phosphates such as  $IP_3$ ), an increase in the peptide activity, thereby determining  
10 whether the ligand is a peptide agonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the peptide according to the invention, which comprises contacting a cell transfected with a vector expressing the  
15 nucleic acid molecule encoding said peptide with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a modification in second messenger concentration  
20 (preferably calcium ions or inositol phosphates such as  $IP_3$ ) or a modification in the cellular metabolism (preferably determined by the acidification rate of the culture medium), a decrease in the peptide activity, thereby determining whether the ligand is a peptide  
25 antagonist.

The present invention relates to a method for determining whether a ligand is an antagonist of the peptide according to the invention, which comprises preparing a cell extract from cells transfected with an  
30 expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cells extract, contacting the membrane fraction with the ligand in the

presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a modification in the production of a second messenger, a decrease in the peptide activity, thereby determining whether the ligand is a peptide antagonist.

Preferably, the second messenger assay comprises measurement of calcium ions or inositol phosphates such as IP<sub>3</sub>.

10 Preferably, the cell used in said method is a mammalian cell non neuronal in origin, such as CHO-K1, HEK293, BHK21, COS-7 cells.

In said method, the ligand is not previously known.

15 The invention is also related to the ligand isolated and detected by any of the preceding methods.

The present invention concerns also the pharmaceutical composition which comprises an effective amount of an agonist or an antagonist of the peptide according to the invention, effective to reduce the activity of said peptide and a pharmaceutically acceptable carrier.

20 It is meant by "an agonist or an antagonist of the peptide according to the invention", all the agonists or antagonists of the known "natural ligand" of the peptide as above described.

Therefore, the previously described methods may be used for the screening of drugs to identify drugs which specifically bind to the peptide according to the invention.

30 The invention is also related to the drugs isolated and detected by any of these methods.



The present invention concerns also a pharmaceutical composition comprising said drugs and a pharmaceutically acceptable carrier.

The invention is also related to a method of  
5 detecting expression of a peptide according to the invention by detecting the presence of mRNA coding for a peptide, which comprises obtaining total RNA or total mRNA from the cell and contacting the RNA or mRNA so obtained with the nucleic acid probe according to the invention  
10 under hybridising conditions and detecting the presence of mRNA hybridised to the probe, thereby detecting the expression of the peptide by the cell.

Said hybridisation conditions are stringent conditions.

15 The present invention concerns also the use of the pharmaceutical composition according to the invention for the treatment and/or prevention of inflammatory diseases, including rheumatoid arthritis, glomerulonephritis, asthma, idiopathic pulmonary fibrosis  
20 and psoriasis, viral infections including Human Immunodeficiency Viruses 1 and 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis and/or auto-immune disorders.

The present invention concerns also a method  
25 for diagnosing a predisposition or a resistance to a disorder associated with the activity of the peptide according to the invention and/or associated with infectious agents such as HIV viruses in a subject. Said method comprises :

30 a) obtaining nucleic acid molecules encoding the peptide according to the invention from the cells of the subject;

- b) possibly performing a restriction digest of said nucleic acid molecules with a panel of restriction enzymes;
- c) possibly electrophoretically separating the resulting nucleic acid fragments on a sized gel;
- d) contacting the resulting gel or the obtained nucleic acid molecule with a nucleic acid probe labelled with a detectable marker and capable of specifically hybridising to said nucleic acid molecule (said hybridisation being made in stringent hybridisation conditions);
- e) detecting labelled bands or the in situ nucleic acid molecules which have hybridised to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern or an in situ marking specific to the subject;
- f) preparing other nucleic acid molecules encoding the peptide according to the invention obtained from the cells of other patients for diagnosis by step a-e; and
- g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby a predisposition or a resistance to the disorder if the patterns are the same or different.

The present invention is also related to a method for diagnosing a predisposition or a resistance to a disorder associated with the activity of a specific allele of the peptide according to the invention or the presence of said peptide at the surface of cells and/or associated with infectious agents such as HIV viruses present in a

subject. Said method comprises :

- a) obtaining a sample of a body fluid, preferably a blood sample comprising antigen presenting cells, from a subject;
- 5 b) adding to said sample a ligand and/or an anti-ligand according to the invention;
- c) detecting the cross-reaction between said ligand and/or said anti-ligand and the specific peptide according to the invention; and
- 10 d) determining whether the peptide corresponds to a receptor or an inactive receptor according to the invention and diagnosing thereby a predisposition or a resistance to the disorder according to the type of the peptide present in the body fluid of the subject.

15           The present invention concerns also a diagnostic and/or dosage device, preferably a kit, comprising the peptides, the nucleic acid molecules, the nucleic acid probes, the ligands and/or the anti-ligands according to the invention, their portions (such as  
20 primers, probes, epitopes, ...) or a mixture thereof, being possibly labelled with a detectable marker.

Said diagnostic and/or dosage device comprises also the reactants for the detection and/or the dosage of antigens, antibodies or nucleic acid sequences  
25 through a method selected from the group consisting of in situ hybridisation, hybridisation or recognition by marked specific antibodies, specially ELISA<sup>®</sup> (Enzyme Linked Immunosorbent Assay) or RIA<sup>®</sup> (Radio Immunoassay), methods on filter, on a solid support, in solution, in "sandwich",  
30 on gel, by Dot blot hybridisation, by Northern blot hybridisation, by Southern blot hybridisation, by isotopic or non-isotopic labelling (such as immunofluorescence or

biotinylation), by a technique of cold probes, by genetic amplification, particularly PCR, LCR, NASBA or CPR, by a double immunodiffusion, by a counter-immunoelectrophoresis, by haemagglutination and/or a mixture thereof.

5 A last aspect of the present invention concerns a method of preparing peptides according to the invention, which comprises :

- 10 a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said peptide so as to permit expression thereof, wherein the cell is preferably selected from the group consisting of bacterial cells, yeast cells, insect  
15 cells and mammalian cells;
- b) inserting the vector of step a in a suitable host cell;
- c) incubating the cell of step b under conditions allowing the expression of the peptide according to the invention;
- 20 d) recovering the peptide so obtained; and
- e) purifying the peptide so recovered, thereby preparing an isolated peptide according to the invention.

The deposits of micro-organisms AchCCR5-SAB1A7 and CHO-K1-pEFIN hCCR5-1/16 were made according to  
25 the Budapest Treaty in the Belgium Coordinated Collection of Micro-organisms (BCCM), Laboratorium voor Moleculaire Biologie (LMBP), Universiteit Gent, K. L. Ledeganckstraat 35, B-9000 GENT, BELGIUM.

30 Short description of the drawings.

The figure 1 represents the primary structure of the peptides according to the invention.

The figure 2 represents the amino acids sequence of the active human CCR5 chemokine receptor according to the invention aligned with that of the human CCR1, CCR2b, CCR3 and CCR4 receptors. Amino acids identical with the active CCR5 sequence are boxed.

The figure 3 shows the chromosomal organisation of the human CCR2 and CCR5 chemokine receptor genes.

The figure 4 shows the functional expression of the human active CCR5 receptor in a CHO-K1 cell line.

The figure 5 represents the distribution of mRNA encoding the CCR5 receptor in a panel of human cell lines of haematopoietic origin.

The figure 6 represents the structure of the mutant form of human CCR5 receptor.

The figure 7 represents the quantification of ENV proteins-mediated fusion by luciferase assays.

The figure 8 represents genotyping of individuals by PCR and segregation of the CCR5 alleles in CEPH families.

The figure 9 represents the FACS analysis of sera anti-CCR5 on a CCR5-CHO cell line according to the invention.

The figure 10 represents the inhibition of HIV infectivity with anti-CCR5 antibodies.

#### Detailed description of the invention.

##### 1. EXPERIMENTALS

##### Materials

Recombinant human chemokines, including MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, IL-8 and GRO $\alpha$  were obtained

from R & D Systems (London, UK). [<sup>125</sup>I]MIP-1α (specific activity, 2200 Ci/mmol) was obtained from Dupont NEN (Brussels, Belgium). Chemokines obtained from R & D Systems were reported by the supplier as >97 % pure on SDS-PAGE  
5 (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and biologically active on a bioassay specific for each ligand. The lyophilised chemokines were dissolved as a 100 µg/ml solution in a sterile phosphate-buffered saline (PBS) and this stock solution was stored at  
10 -20° C in aliquots. Chemokines were diluted to the working concentration immediately before use. All cell lines used in the present study were obtained from the ATCC (Rockville, MD, USA).

#### 15 Cloning and sequencing

The mouse MOP020 clone was obtained by low stringency polymerase chain reaction, as described previously [24, 34], using genomic DNA as template. A human genomic DNA library (Stratagene, La Jolla, CA) constructed  
20 in the lambda DASH vector was screened at low stringency [39] with the MOP020 (511 bp) probe. The positive clones were purified to homogeneity and analysed by Southern blotting. The restriction map of the locus was determined and a relevant XbaI fragment of 4,400 bp was subcloned in  
25 pBluescript SK+ (Stratagene). Sequencing was performed on both strands after subcloning in M13mp derivatives, using fluorescent primers and an automated DNA sequencer (Applied Biosystem 370A). Sequence handling and data analysis was carried out using the DNASIS/PROSIS software (Hitachi), and  
30 the GCG software package (Genetics Computer Group, Wisconsin).

cold buffer, and immediate collection of cells by vacuum filtration through GF/B glass fiber filters (Whatmann) pre-soaked in 0.5 % polyethylenimine (Sigma). Filters were washed three times with 4 ml ice-cold buffer and counted in a gamma counter.

#### Biological activity

The CHO-K1 cell lines stably transfected with the pcDNA3/CCR5 construct or wild type CHO-K1 cells (used as controls) were plated onto the membrane of Transwell cell capsules (Molecular Devices), at a density of  $2.5 \times 10^5$  cells/well in Ham's F12 medium. The next day, the capsules were transferred in a microphysiometer (Cytosensor, Molecular Devices), and the cells were allowed to equilibrate for approximately two hours by perfusion of 1 mM phosphate-buffered (pH 7.4) RPMI-1640 medium containing 0.2 % BSA. Cells were then exposed to various chemokines diluted in the same medium, for a 2 min duration. Acidification rates were measured at one minute intervals.

#### Northern blotting

Total RNA was isolated from transfected CHO-K1 cell lines, from a panel of human cell lines of haematopoietic origin and from a panel of dog tissues, using the RNeasy kit (Qiagen). RNA samples (10 µg per lane) were denatured in presence of glyoxal [26], fractionated on a 1 % agarose gel in a 10 mM phosphate buffer (pH 7.0), and transferred to nylon membranes (Pall Biodyne A, Glen Cove, NY) as described [42]. After baking, the blots were prehybridised for 4h at 42° C in a solution consisting of 50 % formamide, 5x Denhardt solution (1x Denhardt: 0.02 % Ficoll, 0.02 % polyvinylpyrrolidone, 0.02 % BSA), 5x SSPE

(1x SSPE: 0.18 M NaCl, 10 mM Na phosphate, 1 mM EDTA pH 8.3), 0.3 % Sodium Dodecyl Sulphate (SDS), 250 µg per ml denatured DNA from herring testes. DNA probes were ( $\alpha^{32}\text{P}$ )-labelled by random priming [14]. Hybridisations were carried out for 12h at 42° C in the same solution containing 10 % (wt/vol) dextran sulphate and the heat denatured probe. Filters were washed up to 0.1x SSC (1x SSC: 150 mM NaCl, 15 mM Na Citrate pH 7.0), 0.1 % SDS at 60° C and autoradiographed at - 70° C using Amersham  $\beta$ -max films.

## 2. RESULTS AND DISCUSSION

### Cloning and structural analysis

The sequence homology characterising genes encoding G protein-coupled receptors has allowed the cloning by low stringency polymerase chain reaction (PCR) of new members of this gene family [24, 34]. One of the clones amplified from mouse genomic DNA, named MOP020 presented strong similarities with characterised chemokine receptors, sharing 80 % identity with the MCP-1 receptor (CCR2) [8], 65 % identity with the MIP-1 $\alpha$ /RANTES receptor (CCR1) [31], and 51 % identity with IL-8 receptors [20, 30]. The clone was used as a probe to screen a human genomic library. A total of 16 lambda phage clones were isolated. It was inferred from the restriction pattern of each clone and from partial sequence data that all clones were belonging to a single contig in which two different coding sequences were included. One of the coding sequences was identical to the reported cDNA encoding the CCR2 receptor [8, 44]. A 4.400 pb XbaI fragment of a representative clone containing the second region of hybridisation was subcloned in pBluescript SK+. Sequencing



revealed a novel gene, tentatively named CCR5, sharing 84 % identity with the MOP020 probe, suggesting that MOP020 is the mouse ortholog of CCR5. MOP020 does not correspond to any of the three mouse chemokine receptor genes cloned recently [16], demonstrating the existence of a fourth murine chemokine receptor.

The sequence of CCR5 revealed a single open reading frame of 352 codons encoding a protein of 40,600 Da. The sequence surrounding the proposed initiation codon is in agreement with the consensus as described by Kozak [22], since the nucleotide in -3 is a purine. The hydropathy profile of the deduced amino acid sequence is consistent with the existence of 7 transmembrane segments. Alignment of the CCR5 amino acid sequence with that of other functionally characterised human CC-chemokine receptors is represented in figure 2. The highest similarity is found with the CCR2 receptor [8] that shares 75.8 % identical residues. There is also 56.3 % identity with the CCR1 receptor [31], 58.4 % with the CCR3 [10], and 49.1% with the CCR4 [37]. CCR5 represents therefore a new member of the CC-chemokine receptor group [30]. Like the related CCR1 and IL-8 receptors [20, 29, 31, 16] the coding region of CCR5 appears as intronless. From our partial sequencing data, the CCR2 gene is also devoid of intron in the first two thirds of its coding sequence.

Sequence similarities within the chemokine receptor family are higher in the transmembrane-spanning domains, and in intracellular loops. As an example, the identity score between CCR5 and CCR2 goes up to 92% when considering the transmembrane segments only. Lower similarities are found in the N-terminal extracellular domain, and in the extracellular loops. The N-terminal

domain of the IL-8 and CCR2 receptors has been shown to be essential for interaction with the ligand [19, 18]. The variability of this region among CC-chemokine receptors presumably contributes to the specificity towards the various ligands of the family.

A single potential site for N-linked glycosylation was identified in the third extracellular loop of CCR5 (figure 1). No glycosylation site was found in the N-terminal domain of the receptor, where most G protein-coupled receptors are glycosylated. The other chemokine receptors CCR1 and CCR2 present such an N-linked glycosylation site in their N-terminal domain [31, 8]. By contrast, the CCR3 receptor [10] does not display glycosylation sites neither in the N-terminus, nor in extracellular loops. The active CCR5 receptor has four cysteines in its extracellular segments, and all four are conserved in the other CC- and CXC-chemokine receptors (figure 2). The cysteines located in the first and second extracellular loops are present in most G protein-coupled receptors, and are believed to form a disulphide bridge stabilising the receptor structure [41]. The two other cysteines, in the N-terminal segment, and in the third extracellular loop could similarly form a stabilising bridge specific to the chemokine receptor family. The intracellular domains of CCR5 do not include potential sites for phosphorylation by protein kinase C (PKC) or protein kinase A. PKC sites, involved in heterologous desensitisation are frequent in the third intracellular loop and C-terminus of G protein-coupled receptors. CCR1 is also devoid of PKC sites. In contrast, all CC-chemokine receptors, are rich in serine and threonine residues in the C-terminal domain. These residues represent potential

phosphorylation sites by the family of G protein-coupled receptor kinases, and are probably involved in homologous desensitisation [41]. Five of these S/T residues are perfectly aligned in all five receptors (figure 2).

5

#### Physical linkage of the CCR5 and CCR2 genes

As stated above, the 16 clones isolated with the MOP020 probe corresponded to a single contig containing the CCR5 and CCR2 genes. The organisation of this contig was investigated in order to characterise the physical linkage of the two receptor genes in the human genome. A combination of restriction mapping, Southern blotting, fragment subcloning and partial sequencing allowed to determine the respective borders and overlaps of all clones. Out of the 16 clones, 9 turned out to be characterised by a specific restriction map, and their organisation is depicted in figure 3. Four of these clones (#11, 18, 21, 22) contained the CCR2 gene alone, four clones (# 7, 13, 15, 16) contained the ChemR13 gene alone and one clone (#9) contains part of both coding sequences. The CCR2 and CCR5 genes are organised in tandem, CCR5 being located downstream of CCR2. The distance separating CCR2 and CCR5 open reading frames is 17.5 kb. The chromosomal localisation of the tandem is presently unknown. Other chemokine receptors have however been located in the human genome: the CCR1 gene was localised by fluorescence in situ hybridisation to the p21 region of human chromosome 3 [16]. The two IL-8 receptor genes, and their pseudogene have been shown to be clustered on the human 2q34-q35 region [1].

30

#### Functional expression and pharmacology of the active CCR5 receptor

Stable CHO-K1 cell lines expressing the active CCR5 receptor were established and were screened on the basis of the level of CCR5 transcripts as determined by Northern blotting. Three clones were selected and tested for biological responses in a microphysiometer, using various CC- and CXC-chemokines as potential agonists. Wild type CHO-K1 cells were used as control to ensure that the observed responses were specific for the transfected receptor, and did not result from the activation of endogenous receptors. The microphysiometer allows the real time detection of receptor activation, by measuring the modifications of cell metabolism resulting from the stimulation of intracellular cascades [33]. Several studies have already demonstrated the potential of microphysiometry in the field of chemokine receptors. Modifications of metabolic activity in human monocytes, in response CC-chemokines, were monitored using this system [43]. Similarly, changes in the acidification rate of THP-1 cells (a human monocytic cell line) in response to MCP-1 and MCP-3 have been measured [36]. The estimation of the  $EC_{50}$  for both proteins, using this procedure, was in agreement with the values obtained by monitoring the intracellular calcium in other studies [8, 15].

Ligands belonging to the CC- and CXC-chemokine classes were tested on the CCR5 transfected CHO-K1 cells. Whereas MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES were found to be potent activators of the new receptor (figure 4), the CC-chemokines MCP-1, MCP-2 and MCP-3, and the CXC-chemokines GRO $\alpha$  and IL-8 had no effect on the metabolic activity, even at the highest concentrations tested (30 nM). The biological activity of one of the chemokines inducing no response on CCR5 (IL-8) could be demonstrated

on a CHO-K1 cell line transfected with the IL-8A interleukin receptor (Mollereau et al., 1993) : IL-8 produced a 160 % increase in metabolic activity as determined using the microphysiometer. The biological activity of the MCP-2 and MCP-3 preparations as provided by J. Van Damme have been widely documented [2, 40]. MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES were tested on the wild type CHO-K1 cells, at a 30 nM-concentration, and none of them induced a metabolic response. On the CCR5 transfected CHO-K1 cell line, all three active ligands (MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES) caused a rapid increase in acidification rate, reaching a maximum by the second or third minute after perfusion of the ligand. The acidification rate returned to basal level within 10 minutes. The timing of the cellular response is similar to that observed for chemokines on their natural receptors in human monocytes [43]. When agonists were applied repeatedly to the same cells, the response was strongly reduced as compared to the first stimulation, suggesting the desensitisation of the receptor. All measurements were therefore obtained on the first stimulation of each capsule.

The concentration-effect relation was evaluated for the three active ligands in the 0.3 to 30 nM range (figure 3B and C). The rank order of potency was MIP-1 $\alpha$  > MIP-1 $\beta$  = RANTES. At 30 nM concentrations, the effect of MIP-1 $\alpha$  appeared to saturate (at 156 % of baseline level) while MIP-1 $\beta$  and RANTES were still in the ascending phase. Higher concentrations of chemokines could however not be used. The EC50 was estimated around 3 nM for MIP-1 $\alpha$ . The concentrations necessary for obtaining a biological response as determined by using the microphysiometer are in

the same range as those measured by intracellular calcium mobilisation for the CCR1 [31], the CCR2A and B [8], and the CCR3 [10] receptors. The ligand specificity of CCR5 is similar to that reported for CCR3 [10]. CCR3 was described  
5 as the first cloned receptor responding to MIP-1 $\beta$ . However, MIP-1 $\beta$  at 10 nM elicits a significant effect on the CCR5, while the same concentration is without effect on the CCR3 transfected cells [10]. These data suggest that CCR5 could be a physiological receptor for MIP-1 $\beta$ .

10 Binding experiments using [ $^{125}$ I]-human MIP-1 $\alpha$  as ligand did not allow to demonstrate specific binding to CCR53 expressing CHO-K1 cells, using as much as 0.4 nM radioligand and 1 million transfected cells per tube. Failure to obtain binding data could be attributed to a  
15 relatively low affinity of the receptor for MIP-1 $\alpha$ .

#### Northern blotting analysis

Northern blotting performed on a panel of dog tissues did not allow to detect transcripts for CCR5. Given  
20 the role of the chemokine receptor family in mediating chemoattraction and activation of various classes of cells involved in inflammatory and immune responses, the probe was also used to detect specific transcripts in a panel of human cell lines of haematopoietic origin (figure 5). The  
25 panel included lymphoblastic (Raji) and T lymphoblastic (Jurkat) cell lines, promyeloblastic (KG-1A) and promyelocytic (HL-60) cell lines, a monocytic (THP-1) cell line, an erythroleukemia (HEL 92.1.7) cell line, a megakaryoblastic (MEG-01) cell line, and a myelogenous  
30 leukaemia (K-562) cell line. Human peripheral blood mononuclear cells (PBMC), including mature monocytes and lymphocytes, were also tested. CCR5 transcripts (4.4 kb)

could be detected only in the KG-1A promyeloblastic cell line, but were not found in the promyelocytic cell line HL-60, in PBMC, or in any of the other cell lines tested. These results suggest that the active CCR5 receptor could  
5 be expressed in precursors of the granulocytic lineage. CC-chemokines have been reported to stimulate mature granulocytes [27, 38, 23, 2]. However, recent data have also demonstrated a role of CC- and CXC-chemokines in the regulation of mouse and human myeloid progenitor cell  
10 proliferation [6, 7].

CCR5 was shown to respond to MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES, the three chemokines identified as the major HIV-suppressive factors produced by CD8<sup>+</sup> T cells [9], and released in higher amounts by CD4<sup>+</sup> T lymphocytes from  
15 uninfected but multiply exposed individuals [51]. CCR5 represents a major co-receptor for macrophage-tropic (M-tropic) HIV-1 primary isolates and strains [45, 50]. M-tropic strains predominate during the asymptomatic phase of the disease in infected individuals, and are considered as  
20 responsible for HIV-1 transmission. Strains adapted for growth in transformed T-cell lines (T-tropic strains) use as a co-receptor LESTR (or fusin) [50], an orphan receptor also belonging to the chemokine receptor family, but not yet characterised functionally [21, 52, 53]. Dual-tropic  
25 viruses, which may represent transitional forms of the virus in late stages of infection [54] are shown to use both CCR5 and LESTR as co-receptors, as well as the CC-chemokine receptors CCR2b and CCR3 [47]. The broad spectrum of co-receptor usage of dual-tropic viruses suggests that  
30 within infected individuals, the virus may evolve at least in part from selection by a variety of co-receptors expressed on different cell types.

Identification of an inactive  $\Delta$ CCR5 receptor

It is known that some individuals remain uninfected despite repeated exposure to HIV-1 [55, 56, 51].

5 A proportion of these exposed-uninfected individuals results from the relatively low risk of contamination after a single contact with the virus, but it has been postulated that truly resistant individuals do exist. In fact, CD4<sup>+</sup> lymphocytes isolated from exposed-uninfected individuals

10 are highly resistant to infection by primary M-tropic, but not T-tropic HIV-1 strains. Also, peripheral blood mononuclear cells (PBMC) from different donors are not infected equally with various HIV-1 strains [57-59]. Given the key role played by CCR5 in the fusion event that

15 mediates infection by M-tropic viruses, it is postulated that variants of CCR5 could be responsible for the relative or absolute resistance to HIV-1 infection exhibited by some individuals, and possibly for the variability of disease progression in infected patients [66]. The Inventors

20 selected three HIV-1 infected patients known to be slow progressors, and four seronegative individuals as controls; the full coding region of their CCR5 gene was amplified by PCR and sequenced. Unexpectedly, one of the slow progressors, but also two of the uninfected controls,

25 exhibited heterozygosity at the CCR5 locus for a biallelic polymorphism. The frequent allele corresponded to the published CCR5 sequence, while the minor one displayed a 32 bp deletion within the coding sequence, in a region corresponding to the second extracellular loop of the

30 receptor (Fig. 6). The figure 6 is the structure of the mutant form of human CC-chemokine receptor 5. a, The amino acid sequence of the non-functional  $\Delta$ CCR5 protein is



represented. The transmembrane organisation is given by analogy with the predicted transmembrane structure of the wild-type CCR5. Amino acids represented in black correspond to unnatural residues resulting from the frame shift caused by the deletion. The mutant protein lacks the last three transmembrane segments of CCR5, as well as the regions involved in G protein-coupling. b, Nucleotide sequence of the CCR5 gene surrounding the deleted region, and translation into the normal receptor (top) or the truncated mutant ( ccr5, bottom). The 10-bp direct repeat is represented in italics. The full size coding region of the CCR5 gene was amplified by PCR, using 5'-TCGAGGATCCAAGATGGATTATCAAGT-3' and 5'-CTGATCTAGAGCCATGTGCACAACTCT-3' as forward and reverse primers respectively. The PCR products were sequenced on both strands using the same oligonucleotides as primers, as well as internal primers, and fluorochrome-labelled dideoxynucleotides as terminators. The sequencing products were run on an Applied Biosystem sequencer, and ambiguous positions were searched along the coding sequence. When the presence of a deletion was suspected from direct sequencing, the PCR products were cloned after restriction with *Bam*HI and *Xba*I endonucleases into pcDNA3. Several clones were sequenced to confirm the deletion. The deletion was identical in three unrelated individuals investigated by sequencing.

Cloning of the PCR product and sequencing of several clones confirmed the deletion. The deletion causes a frame shift, which is expected to result in premature termination of translation. The protein encoded by this mutant allele ( $\Delta$ ccr5) therefore lacks the last three transmembrane segments of the receptor. A 10-bp direct

repeat flanking the deleted region (Fig. 6b) on both sides is expected to have promoted the recombination event leading to the deletion. Numerous mutagenesis studies performed on various classes of G protein-coupled  
5 receptors, including chemokine receptors, makes it clear that such a truncated protein is certainly not functional in terms of chemokine-induced signal transduction: it lacks the third intracellular loop and C-terminal cytoplasmic domains, the two regions involved primarily in G protein  
10 coupling [41]. In order to test whether the truncated protein was able to function as a HIV-1 co-receptor, the Inventors tested its ability to support membrane fusion by both primary M-tropic and dual-tropic virus ENV proteins. The recombinant protein was expressed in quail QT6 cells  
15 together with human CD4. The QT6 cells were then mixed with HeLa cells expressing the indicated viral ENV protein and the extent of cell-cell fusion measured using a sensitive and quantitative gene-reporter assay. In contrast to wild-type CCR5, the truncated receptor did not allow fusion with  
20 cells expressing the ENV protein from either M-tropic or dual-tropic viruses (Figure 7). The figure 7 represents the quantification of ENV protein-mediated fusion by luciferase assay. To quantify cell-cell fusion events, Japanese quail QT6 fibrosarcoma cells were transfected or cotransfected as  
25 indicated with the pCDNA3 vector (Invitrogen) containing the coding sequence for wild-type CCR5, the truncated ccr5 mutant, the CCR2b or the Duffy chemokine receptors, or with the pCDNA3 vector alone. The target cells were also transfected with human CD4 expressed from the CMV promoter  
30 and the luciferase gene under the control of the T7 promoter. HeLa effector cells were infected (MOI = 10) with vaccinia vectors expressing T7-polymerase (vTF1.1) and

either the JR-FL (vCB28) or 89.6 (vBD3) envelope proteins. The luciferase activity resulting from cell fusion is expressed as the percentage of the activity (in relative light units) obtained for wild-type CCR5. All transfections  
5 were performed with an identical quantity of plasmid DNA using pcDNA3 as carrier when necessary. To initiate fusion, target and effector cells were mixed in 24 well plates at 37 °C in the presence of ara-C and rifampicin, and allowed to fuse for 8 hours. Cells were lysed in 150 µl of reporter  
10 lysis buffer (Promega) and assayed for luciferase activity according to the manufacturer's instructions (Promega).

Coexpression of  $\Delta$ CCR5 with wild-type CCR5 consistently reduced the efficiency of fusion for both JR-FL and 89.6 envelopes, as compared with CCR5 alone. Whether  
15 this in vitro inhibitory effect (not shared by the chemokine receptor Duffy, used as control) also occurs in vivo is presently not known. Coexpression with the CCR2b receptor [31], which is the CC-chemokine receptor most closely related to CCR5 but does not promote fusion by M-  
20 tropic HIV-1 strains [48], did not rescue the mutation by formation of a hybrid molecule (Fig. 7).

The figure 8 represents genotyping of individuals by PCR and segregation of the CCR5 alleles in CEPH families. a, Autoradiography illustrating the pattern  
25 resulting from PCR amplification and EcoRI cleavage for individuals homozygous for the wild-type CCR5 allele (CCR5/CCR5), the null  $\Delta$ CCR5 allele ( $\Delta$ CCR5/ $\Delta$ CCR5), and for heterozygotes (CCR5/ $\Delta$ CCR5). A 735 bp PCR product is cleaved into a common band of 332 bp for both alleles, and into 403  
30 and 371 bp bands for the wild-type and mutant alleles, respectively. b, Segregation of the CCR5 alleles in two informative families of the CEPH. Half-black and white

symbols represent heterozygotes and wild-type homozygotes, respectively. For a few individuals in the pedigrees, DNA was not available (ND: not determined). PCRs were performed on genomic DNA samples, using 5'-CCTGGCTGTCGTCCATGCTG-3' and 5'-CTGATCTAGAGCCATGTGCACAACTCT-3' as forward and reverse primers respectively. Reaction mixtures consisted in 30  $\mu$ l of 10 mM Tris-HCl buffer pH 8.0, containing 50 mM KCl, 0.75 mM MgCl<sub>2</sub>, 0.2 mM dCTP, dGTP and dTTP, 0.1 mM dATP, 0.5  $\mu$ i [ $\alpha$ -<sup>32</sup>P]-dATP, 0.01% gelatine, 5% DMSO, 200 ng target DNA, 60 ng of each of the primers and 1.5 U Taq polymerase. PCR conditions were: 93 °C for 2 min 30; 93 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, 30 cycles; 72 °C for 6 min. After the PCR reaction, the samples were incubated for 60 min at 37 °C with 10 U EcoRI, and 2  $\mu$ l of the denatured reaction mixture was applied onto a denaturing 5% polyacrylamide gel containing 35% formamide and 5.6 M urea. Bands were detected by autoradiography.

Based on the 14 chromosomes tested in the first experiment, the deleted  $\Delta$ CCR5 allele appeared rather frequent in the Caucasian population. The accurate frequency was further estimated by testing (Fig. 8a) a large cohort of Caucasian individuals, including unrelated members of the CEPH (Centre d'Etude des Polymorphismes Humains) families, part of the IRIBHN staff, and a bank of anonymous DNA samples from healthy individuals collected by the Genetics Department of the Erasme Hospital in Brussels. From a total of more than 700 healthy individuals, the allele frequencies were found to be 0.908 for the wild-type allele, and 0.092 for the mutant allele (Table I). The genotype frequencies observed in the population were not significantly different from the expected Hardy-Weinberg distribution (CCR5/CCR5: 0.827 vs 0.324; CCR5/ $\Delta$ CCR5: 0.162

vs 0.167;  $\Delta\text{CCR5}/\Delta\text{CCR5}$ : 0.011 vs 0.008,  $p > 0.999$ ), suggesting that the null allele has no drastic effect on fitness. Using two informative CEPH families, it was confirmed that the wild-type CCR5 gene and its  $\Delta\text{CCR5}$  variant were allelic, and segregated in a normal mendelian fashion (Fig. 8b). Interestingly, a cohort of 124 DNA samples originating from Central Africa (collected from Zaire, Burkina Fasso, Cameroun, Senegal and Benin) and Japan did not reveal a single  $\Delta\text{CCR5}$  mutant allele, suggesting that this allele is either absent or very rare in Asian, African black populations (Table I).

The consequences of the existence of a null allele of CCR5 in the normal Caucasian population were then considered in terms of susceptibility to infection by HIV-1. If, as it is predicted, CCR5 plays a major (not redundant) role in the entry of most primary virus strains into cells, then  $\Delta\text{CCR5}/\Delta\text{CCR5}$  individuals should be particularly resistant to HIV-1 challenge, both in vitro and in vivo. The frequency of the  $\Delta\text{CCR5}/\Delta\text{CCR5}$  genotype should therefore be significantly lower in HIV-1 infected patients, and increased in exposed-uninfected individuals. Also, if heterozygotes have a statistical advantage due to the lower number of functional receptors on their white blood cells, or to the possible dominant-negative properties of the mutant allele, the frequency of heterozygotes (and mutant alleles) should be decreased in HIV-infected populations. These hypotheses were tested by genotyping a large number of seropositive Caucasian individuals ( $n = 645$ ) belonging to cohorts originating from various hospitals from Brussels, Liège and Paris (Table I). Indeed, it was found that within this large series, the

frequency of the null *Accr5* allele was significantly reduced from 0.092 to 0.053 ( $p < 10^{-5}$ ). The frequency of heterozygotes was also reduced from 0.162 to 0.106 ( $p < 0.001$ ) and not a single *Accr5/Accr5* individual could be found ( $p < 0.01$ ).

Altogether, functional and statistical data suggest that CCR5 is indeed the major co-receptor responsible for natural infection by M-tropic HIV-1 strains. Individuals homozygous for the null *Accr5* allele (about 1% of the Caucasian population) have apparently a strong resistance to infection. It is unclear at this point whether resistance to HIV-1 is absolute or relative, and whether resistance will vary depending on the mode of viral contamination. Larger cohorts of seropositive individuals will have to be tested in order to clarify this point. Heterozygotes have a milder though significant advantage: assuming an equal probability of contact with HIV, it can be inferred from Table I that heterozygotes have a 39% reduction in their likeliness of becoming seropositive, as compared to individuals homozygous for the wild-type CCR5 allele. Both a decrease in functional CCR5 receptor number, and a dominant-negative effect of *Accr5* *in vivo*, comparable to what is observed in the *in vitro* experiments (Fig. 7) are possible explanations for this relative protection. The mutant allele, which can be regarded as a natural knock-out in human, is not accompanied by an obvious phenotype in homozygous individuals. Nevertheless, the lack of overt phenotype, taken together with the relative protection that characterises heterozygous subjects, suggests that pharmacological agents that selectively block the ability of HIV-1 to utilise CCR5 as a cofactor, could be effective

in preventing HIV-1 infection, and would be predicted not be associated with major side effects resulting from CCR5 inactivation. These pharmaceutical agents could be used with other compounds which are able to block other chemokine receptors used as co-receptors by some HIV-primary isolates in order to infect other cells [47]. The prevalence of the null allele in the Caucasian population raises the question of whether pandemic of HIV (or related viruses using the same co-receptor) have contributed during mankind's evolution to stabilise by selection the mutant ccr5 allele at such a high frequency.

#### Production of antibodies anti-CCR5

Antibodies were produced by genetic immunisation. Six week old females balb/c mice were used. DNA coding for the human CCR5 receptor was inserted in the expression vector pcDNA3 under the control of the CMV promotor and 100 µg DNA was injected in the anterior tibial muscle, five days after pre-treatment of this muscle with cardiotoxine (from venom of Naja Nigricolis). Injections were repeated twice at three week intervals. Fifteen days after the last injection, blood was taken from each animal and sera were tested for the presence of anti-CCR5 antibodies.

25

#### Test of sera using Fluorescence Activated Cell Sorter (FACS)

Sera were tested by fluorescence activated cell sorting using recombinant CHO cells expressing the CCR5 receptor. Briefly, cells were detached using a PBS-EDTA-EGTA solution and incubated into PBS-BSA medium for 30 minutes at room temperature with 5 µl serum on the basis of

100,000 cells per tube. Cells were then washed and incubated for 30 minutes in ice together with anti-mouse antibody labelled with fluorescein. Cells were washed, taken up into 200  $\mu$ l of a PBS-BSA solution and fluorescence  
5 was analysed by FACS (FACSCAN, Becton-Dickinson). 10,000 cells were counted. Wild type CHO or recombinant CHO cells expressing the human CCR2b receptor were used as controls.

When tested by FACS analysis 2 weeks after the last injection (figure 9), all the sera from mice  
10 immunised with CCR5 cDNA, clearly recognised the native receptor expressed on CHO cells (mean of fluorescence = 200), without significant cross reaction with control cells expressing CCR2b (mean of fluorescence = 20).

Sera were tested on either a CHO cell line  
15 expressing high level of CCR5 receptor (black histogram) or a CHO cell line expressing CCR2b receptor (white histogram) as negative control. Each serum was tested individually.

#### Antibodies anti-CCR5 and HIV infectivity

20 Peripheral blood mononuclear cells (PBMC) from one donor homozygous for wild type CCR5 gene, were isolated and cultivated 3 days in presence of PHA.

On day 4, 800  $\mu$ l of cells ( $10^5$  cells/ml) were incubated with 8  $\mu$ l of sera from mice immunised with CCR5  
25 cDNA, 30 minutes at 37 °C. 1 ml of viral solution (JRCSF HIV strain) is then added and incubated during 2 hours. Cells were then washed twice and cultivated during 15 days.

Aliquot of medium is taken at days 0, 4, 7, 10 and 14 and the dosage of antigen p24 is performed.

30 14 days after the beginning of the experiment, one serum (serum B0) totally block the production of p24, indicating its ability to block the



infection of the lymphocytes by this HIV strain (figure 10). Other serums also exhibit a partial or total effect on this infection (serum A2 and B1). All the other sera did not show any effect on this infection.

5

#### Production of monoclonal antibodies

Mice with the highest titre of CCR5 antibodies were selected for monoclonal antibodies production and injected intravenously with 10<sup>7</sup> recombinant  
10 CHO-K1 cells expressing human CCR5 receptors. Three days later, animals were sacrificed and fusion of splenic cells or cells from lymph nodes near the site of injection with SP2/0 myeloma cells, were performed. Fusion protocol used was that of Galfre et al. (Nature 266, 550 (1977)). A  
15 selective HAT (hypoxanthine/aminopterin/thymidin) medium is used to select hybridomas and their supernatants are tested by FACS using recombinant CHO cells expressing the human CCR5 receptor, as it was done for the sera. Positives hybridomas are then cloned by limited dilution. Clones that  
20 are shown positive by FACS analyses are then expanded and produced in ascites in balb/C mice.

Table 1

Seronegative				Seropositive			Chi-squared
Number	Frequency	Standard error	Number	Frequency	Standard error		
Genotypes :							2 degrees of freedom 17.7 p < 0.0005
CCR5/CCR5	582	0.827	0.014	645	0.892	0.012	
CCR5/Δ CCR5	114	0.162	0.014	78	0.108	0.012	
CCR5/Δ CCR5	8	0.011	0.004	0	0.000	< 0.001	
Total :	704	1.000		723	1.000		
Alleles :							1 degree of freedom freedom p < 0.0005
CCR5	1278	0.908	0.008	1368	0.946	0.006	
Δ CCR5	130	0.092	0.008	78	0.054	0.006	
Total :	1408	1.000		1446	1.000		

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CLAIMS

1. Peptide having at least an amino acid sequence which presents more than 80% homology with the amino acid sequence as represented in SEQ ID NO. 1 shown in  
5 figures 1.
2. Peptide according to the claim 1, having at least an amino acid sequence which presents more than 90% homology with the amino acid sequence as represented in SEQ ID NO. 1 shown in figures 1.
- 10 3. Peptide according to the claim 1 or 2, having the amino acid sequence of SEQ ID NO. 1 or a portion thereof.
4. Peptide according to any of the claims 1 to 3, having at least an amino acid sequence which  
15 presents more than 80% homology with the amino acid sequence as represented in SEQ ID NO. 2 shown in figures 1.
5. Peptide according to the claim 4, having at least an amino acid sequence which presents more than 90% homology with the amino acid sequence as  
20 represented in SEQ ID NO. 2 shown in figures 1.
6. Peptide according to the claim 4 or 5 having the amino acid sequence of SEQ ID NO. 2 or a portion thereof.
7. Peptide according to any of the claims 1  
25 to 3, having at least an amino acid sequence which presents more than 80% homology with the amino acid sequence as represented in SEQ ID NO. 3 shown in figures 1.
8. Peptide according to the claim 5, having at least an amino acid sequence which presents more  
30 than 90% homology with the amino acid sequence as represented in SEQ ID NO. 3 shown in figures 1.

9. Peptide according to the claim 7 or 8, having the amino acid sequence of SEQ ID NO. 3 or a portion thereof.

10. Peptide according to any of the claims 4 to 6, characterised in that it is a CC chemokine receptor. \*

11. Peptide according to the claim 10, characterised in that the CC chemokine receptor is stimulated by the MIP-1 $\beta$  chemokine at a concentration less or equal to 10 nM.

12. Peptide according to the claim 10 or 11 characterised in that the CC chemokine receptor is stimulated by the MIP-1 $\alpha$  or RANTES chemokines.

13. Peptide according to any of the claims 10 to 12, characterised in that the CC chemokine receptor is not stimulated by the MCP-1, MCP-2, MCP-3, IL-8 and GRO $\alpha$  chemokines.

14. Peptide according to any of the claims 4 to 6 and 10 to 13, characterised in that it is a receptor of HIV-1 and/or HIV-2 viruses or a portion of said HIV viruses.

15. Peptide according to any of the claims 7 to 9, characterised in that it is an inactive CC chemokine receptor.

16. Peptide according to any of the claims 7 to 9 or 15, characterised in that it is an inactive receptor which is not a receptor of HIV-1 and/or HIV-2 viruses or a portion of said HIV viruses.

17. Peptide according to any of the claims 10 to 16, being a human receptor.

18. Nucleic acid molecule having more than 80%, preferably more than 90%, homology with one of the

nucleic acid sequences SEQ ID NO. 1, SEQ ID NO. 2 and SEQ ID NO. 3 shown in figures 1.

19. Nucleic acid molecule according to the claim 18, which has at least the nucleic acid sequence SEQ ID NO. 1, SEQ ID NO. 2 or SEQ ID NO. 3 shown in figure 1 or a portion (such as a probe or a primer) thereof.

20. Nucleic acid molecule encoding a peptide according to any of the claims 1 to 17.

21. Nucleic acid molecule according to any of the claims 18 to 20, which is a cDNA molecule or a genomic DNA molecule.

22. Vector comprising the nucleic acid molecule according to any of the claims 18 to 21.

23. Vector of the claim 22, adapted for expression in a cell, which comprises the regulatory elements necessary for expression of the nucleic acid molecule in said cell operatively linked to the nucleic acid molecule according to any of the claims 18 to 21 as to permit expression thereof.

24. Vector of the claim 23, wherein the cell is chosen among the group consisting of bacterial cells, yeast cells, insect cells or mammalian cells.

25. Vector according to any of the claims 22 to 24, wherein the vector is a plasmid or a virus.

26. Vector according to claim 25 being a virus, chosen among the group consisting of baculoviruses, adenoviruses or semliki forest viruses.

27. Vector of the claim 25, being the pcDNA3 plasmid.

28. Cell, preferably a human cell, comprising the vector according to any of the claims 22 to 27.

29. Cell according to the claim 28,



characterised in that it is transformed also by another vector encoding a protein enhancing the functional response in said cell, preferably said protein being the Gal5 or the Gal6 protein.

5                   30. Cell of the claim 28 or 29, wherein the cell is a mammalian cell, such as a non neuronal cell in origin, which is preferably chosen among the group consisting of CHO-K1, HEK293, BHK21, COS-7 cells.

10                   31. Cell according to the claim 30 being the cell CHO-K1-pEFIN hCCR5-1/16.

                  32. Nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridising with an unique sequence included within the nucleic acid molecule according to any of the  
15                   claims 18 to 21.

                  33. Antisense oligonucleotide having a sequence capable of specifically hybridising to a nucleic acid molecule of the claims 18 to 21, so as to prevent translation of said nucleic acid molecule.

20                   34. Antisense oligonucleotide having a sequence capable of specifically hybridising to the DNA molecule of the claims 20 or 21 or a portion thereof.

                  35. Antisense oligonucleotide according to the claim 33 or 34, comprising chemical analogs of  
25                   nucleotides.

                  36. Ligand capable of binding to the peptide according to any of the claims 1 to 17 with the proviso that it is not a known "natural ligand" of said peptide, which is preferably chosen among the group  
30                   consisting of MIP-1 $\beta$ , MIP-1 $\alpha$  OR RANTES CC-chemokines, HIV viruses or a portion of said HIV viruses.

37. Anti-ligand capable of competitively inhibiting the binding of the known "natural ligand" to the peptide according to any of the claims 1 to 17.

38. Ligand according to the claim 36, which  
5 is an antibody.

39. Anti-ligand according to the claim 37, which is an antibody.

40. Antibody according to the claim 38 or 39, which is a monoclonal antibody.

10 41. Monoclonal antibody according to the claim 40, directed to an epitope of the peptide according to any of the claims 1 to 17, present on the surface of a cell expressing said peptide.

42. Cell producing the monoclonal antibody  
15 according to claim 40 being the cell AchCCR5-SAB1A7.

43. Pharmaceutical composition comprising an amount of the oligonucleotide according to claim 33, effective to decrease activity of the peptide according to any of the claims 1 to 17 by passing through a cell  
20 membrane and binding specifically with mRNA encoding said peptide in the cell so as to prevent its translation, and a pharmaceutically acceptable carrier capable of passing through a cell membrane.

44. Pharmaceutical composition of the claim  
25 43, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

45. Pharmaceutical composition of the claim 44, wherein the substance which inactivates mRNA is a ribozyme.

30 46. Pharmaceutical composition according to any of the claims 43 to 45, wherein the pharmaceutically acceptable carrier comprises a structure which binds to a

receptor on a cell capable of being taken up by cell after binding to the structure.

47. Pharmaceutical composition which comprises an effective amount of the anti-ligand of the claim 37, effective to block binding of a ligand to the peptide according to any of the claims 1 to 17 and a pharmaceutically acceptable carrier.

48. Transgenic non human mammal expressing the nucleic acid molecule according to any of the claims 18 to 21.

49. Transgenic non human mammal comprising an homologous recombination knockout of the native peptide according to any of the claims 1 to 17.

50. Transgenic non human mammal whose genome comprises antisense nucleic acid complementary to the nucleic acid molecule according to any of the claims 18 to 21 so placed as to be transcribed into antisense mRNA which is complementary to the nucleic acid molecule of the claims 18 to 22 and which hybridises to said nucleic acid molecule thereby reducing its translation.

51. Transgenic non human mammal according to any of the claims 48 to 50, wherein the nucleic acid according to any of the claims 18 to 22 additionally comprises an inducible promoter.

52. Transgenic non human mammal according to any of the claims 48 to 51, wherein the nucleic acid according to claim 18 to 21 additionally comprises tissue specific regulatory elements.

53. Transgenic non human mammal according to any of the claims 48 to 52, which is a mouse.

54. Method for determining whether a ligand can specifically bind to a peptide according to any of the

claims 1 to 17, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting binding of ligand to such peptide and  
5 detecting the presence of any such ligand bound specifically to said peptide, thereby determining whether the ligand binds specifically to said peptide.

55. Method for determining whether a ligand can specifically bind to the peptide according to any of  
10 the claims 1 to 17, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the  
15 ligand with the membrane fraction under conditions permitting binding of the ligand to such peptide and detecting the presence of any ligand bound to said peptide, thereby determining whether the compound is capable of specifically binding to said peptide.

56. Method for determining whether a ligand  
20 is an agonist of the peptide according to any of the claims 1 to 17, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand under conditions permitting the activation of a functional receptor response from the cell  
25 and detecting by means of a bio-assay, such as a second messenger response, an increase in the peptide activity, thereby determining whether the ligand is a peptide agonist.

57. Method for determining whether a ligand  
30 is an agonist of the peptide according to any of the claims 1 to 17, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid

molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with the ligand under conditions permitting the activation of a functional peptide response and detecting  
5 by means of a bio-assay, such as a second messenger response, an increase in the peptide activity, thereby determining whether the ligand is a peptide agonist.

58. Method for determining whether a ligand is an antagonist of the peptide according to any of the  
10 claims 1 to 17, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response  
15 and detecting by means of a bio-assay, such as a second messenger response, a decrease in the peptide activity, thereby determining whether the ligand is a peptide antagonist.

59. Method for determining whether a ligand  
20 is an antagonist of the peptide according to any of the claims 1 to 17, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane  
25 fraction with the ligand in the presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response and detecting by means of a bio-assay, such as a second messenger response, a decrease in the peptide activity, thereby determining whether the  
30 ligand is a receptor antagonist.

60. A method according to any of the claims 54 to 59, wherein the second messenger assay comprises

measurement of calcium ions ( $\text{Ca}^{2+}$ ), inositol phosphates (such as  $\text{IP}_3$ ), diacylglycerol (DAG) or cAMP.

61. Method according to any of the claims 54 to 60, wherein the cell is a mammalian cell, preferably non neuronal in origin, and chosen among the group consisting of CHO-K1, HEK293, BHK21 and COS-7 cells.

62. Method according to any of the claims 54 to 61, wherein the ligand is not previously known.

63. Ligand detected by the method according to any of the claims 54 to 62.

64. Pharmaceutical composition which comprises the ligand according to the claim 63 and a pharmaceutically acceptable carrier.

65. Method of screening drugs to identify drugs which specifically bind to the peptide according to any of the claims 1 to 17 on the surface of the cell, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with a plurality of drugs under conditions permitting binding of said drugs to the peptide, and determining those drugs which specifically bind to the transfected cell, thereby identifying drugs which specifically bind to the peptide.

66. Method of screening drugs to identify drugs which specifically bind to the peptide according to any of the claims 1 to 17 on the surface of the cell, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs and determining those drugs which bind to the transfected cell, thereby identifying drugs which

specifically bind to said peptide.

67. Method of screening drugs to identify drugs which act as agonists of the peptide according to any of the claims 1 to 17, which comprises contacting a cell  
5 transfected with a vector expressing the nucleic acid molecule encoding said peptide with a plurality of drugs under conditions permitting the activation of a functional peptide response, and determining those drugs which  
10 activates such peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide agonists.

~~68. Method of screening drugs to identify~~  
drugs which act as agonists of the peptide according to any of the claims 1 to 17, which comprises preparing a cell  
15 extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs under  
20 conditions permitting the activation of a functional peptide response, and determining those drugs which activate such peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide agonists.

69. Method of screening drugs to identify  
25 drugs which act as antagonists of the peptide according to any of the claims 1 to 17, which comprises contacting a cell transfected with a vector expressing the nucleic acid molecule encoding said peptide with a plurality of drugs in  
the presence of a known peptide agonist, under conditions  
30 permitting the activation of a functional peptide response, and determining those drugs which inhibit the activation of the peptide using a bio-assay, such as a second messenger

response, thereby identifying drugs which act as peptide antagonists.

70. Method of screening drugs to identify drugs which act as antagonists of the peptide according to any of the claims 1 to 17, which comprises preparing a cell extract from cells transfected with a vector expressing the nucleic acid molecule encoding said peptide, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a plurality of drugs in presence of a known peptide agonist, under conditions permitting the activation of a functional peptide response, and determining those drugs which inhibit the activation of the peptide using a bio-assay, such as a second messenger response, thereby identifying drugs which act as peptide antagonists.

71. Method according to any of the claims 67 to 70, wherein the functional response detected by means of a bioassay is detected and measured by a microphysiometer.

72. Drug detected by any of the methods according to claims 65 to 71.

73. Pharmaceutical composition comprising a drug according to the claim 72 and a pharmaceutically acceptable carrier.

74. Method of detecting the expression of the peptide according to any of the claims 1 to 17, by detecting the presence of mRNA coding said receptor, which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with the nucleic acid probe according to the claim 32 under hybridising conditions, and detecting the presence of mRNA hybridised to the probe, thereby detecting the expression of the peptide by the cell.



75. Method of detecting the presence of the peptide according to any of the claims 1 to 17 on the surface of a cell, which comprises contacting the cell with the anti-ligand of claim 38 under conditions permitting  
5 binding of the antibody to the peptide, and detecting the presence of the antibody bound to the cell, thereby detecting the presence of the peptide on the surface of the cell.

76. Method of determining the physiological  
10 effects of expressing varying levels of the peptide according to any of the claims 1 to 17, which comprises producing a transgenic non-human mammal according to any of the claims 48 to 53 whose levels of peptide expression are varied by use of an inducible promoter which regulates the  
15 peptide regulation.

77. Method of determining the physiological effects of expressing varying levels of the peptide according to any of the claims 1 to 17, which comprises producing a panel of transgenic non human mammals according  
20 to any of the claims 48 to 53, each expressing a different amount of said peptide.

78. Method for identifying an antagonist of the peptide according to any of the claims 1 to 17 capable of alleviating an abnormality in a subject wherein the  
25 abnormality is alleviated by decreasing the activity of the peptide, which comprises administering the antagonist to a transgenic non human mammal according to any of the claims 48 to 53 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the  
30 transgenic non human mammal as a result of peptide activity, thereby identifying the antagonist.

79. Antagonist identified by any of the methods of the claims 74 to 78.

80. Pharmaceutical composition comprising an antagonist according to the claim 79 and a  
5 pharmaceutically acceptable carrier.

81. Method for identifying an agonist of the peptide according to any of the claims 1 to 17 capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by activation of said peptide,  
10 which comprises administering the agonist to a transgenic non human mammal according to any of the claims 48 to 53 and determining whether the antagonist alleviates the physical and behavioural abnormalities displayed by the transgenic non human mammal, the alleviation of the  
15 abnormalities indicating the identification of the agonist.

82. Agonist identified by the method of the claim 81.

83. Pharmaceutical composition comprising an agonist according to the claim 82 and a pharmaceutically  
20 acceptable carrier.

84. Method for diagnosing a predisposition or a resistance to a disorder associated with the activity of a specific allele of the peptide according to any of the claims 1 to 17, and/or associated with infectious agents,  
25 preferably the HIV-1 and/or HIV-2 viruses, present in a subject, which comprises :

- a) obtaining nucleic acid molecules encoding said peptide from the cells of the subject,
- b) possibly performing a restriction digest of said nucleic  
30 acid molecules with a panel of restriction enzymes,
- c) possibly electrophoretically separating the resulting nucleic acid fragments on a sized gel,

- d) contacting the resulting gel or the obtained nucleic acid molecules labelled with a nucleic acid probe with a detectable marker and capable of specifically hybridising to said nucleic acid molecule,
- 5 e) detecting labelled bands or in situ nucleic acid molecules which have hybridised to the said nucleic acid molecule labelled with a detectable marker to create a unique band pattern or in situ marking specific to the subject,
- 10 f) preparing other nucleic acid molecules encoding said peptide obtained from the cells of other subjects for ~~diagnosis by step a-e; and~~
- g) comparing the unique band pattern specific to the nucleic acid molecule of subjects suffering from the
- 15 disorder from step e and the nucleic acid molecule obtained for diagnosis from step f to determine whether the patterns are the same or different and to diagnose thereby predisposition or resistance to the disorder if the patterns are the same or different.
- 20 85. Method for diagnosing a predisposition or a resistance to a disorder associated with the activity of a specific allele of the peptide according to any of the claims 1 to 17 or the presence of said peptide at the surface of cells, and/or associated with infectious agents,
- 25 preferably the HIV and/or HIV-2 viruses, present in a subject, which comprises :
- a) obtaining a sample of a body fluid, preferably a blood sample comprising antigen presenting cells, from a subject,
- b) adding to said sample a ligand and/or an anti-ligand
- 30 according to any of the claims 36 to 41,
- c) detecting the cross-reaction between said ligand and/or said anti-ligand and the specific peptide, and

d) determining whether the peptide corresponds to a receptor or an inactive receptor and diagnosing thereby a predisposition or a resistance to the disorder according to the type of the peptide present in the body fluid of the  
5 subject.

86. Diagnostic and/or dosage device comprising the peptide according to any of the claims 1 to 17, the nucleic acid molecule according to any of the claims 18 to 21, the nucleic acid probe according to the  
10 claim 33, the ligand and anti-ligand according to any of the claims 36 to 41, the known "natural ligands" their portions (such as primer, probes, epitopes, ...) and/or a mixture thereof, being possibly labelled with a detectable marker.

15 87. Diagnostic and/or dosage device according to the claim 86, characterised in that it comprises the reactants for the detection and/or dosage of antigens, antibodies or nucleic acid sequences through a method selected from the group consisting of in situ  
20 hybridisation, hybridisation or recognition by marked specific antibodies, specially ELISA<sup>®</sup> (Enzyme Linked Immunosorbent Assay) or RIA<sup>®</sup> (Radio Immunoassay), methods on filter, on a solid support, in solution, in "sandwich", on gel, by Dot blot hybridisation, by Northern blot  
25 hybridisation, by Southern blot hybridisation, by isotopic or non-isotopic labelling (such as immunofluorescence or biotinylation), by a technique of cold probes, by genetic amplification, particularly PCR, LCR, NASBA or CPR, by a double immunodiffusion, by a counter-immunoelectrophoresis,  
30 by haemagglutination and/or a mixture thereof.

88. Method of preparing peptides according to any of the claims 1 to 17, which comprises :

- a) constructing a vector adapted for expression in a cell which comprises the regulatory elements necessary for the expression of nucleic acid molecules in the cell operatively linked to nucleic acid molecule encoding said peptide so as to permit expression thereof, wherein the cell is preferably selected from the group consisting of bacterial cells, yeast cells, insect cells and mammalian cells,
- b) inserting the vector of step a in a suitable host cell,
- 10 c) incubating the cell of step b under conditions allowing the expression of the peptide according to the invention,
- d) recovering the peptide so obtained, and possibly
- e) purifying the peptide so recovered.

89. Use of the pharmaceutical composition

15 according to any of the claims 43 to 47, 64, 73, 80 and 83, for the preparation of a medicament in the treatment of a disease chosen from the group consisting of inflammatory diseases, including rheumatoid arthritis, glomerulonephritis, asthma, idiopathic pulmonary fibrosis

20 and psoriasis, viral infections including infections by Human Immunodeficiency Viruses 1 and 2 (HIV-1 and 2), cancer including leukaemia, atherosclerosis and/or autoimmune disorders.

SEQ ID NO.1FIG.1 a

GAATTCCCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
	19
TTCCCTTCACTACAAAACCTTCATTGCTTGGCCAAAAGAGAGTTAATTCAATGTAGACAT	119
	39
CTATGTAGGCAATTAAAAACCTATTGATGTATAAAACAGTTTGCATTTCATGGAGGGCAAC	179
	59
TAAATACATTCTAGGACTTTATAAAAGATCACTTTTTTATTTATGCACAGGGTGGAACAAG	239
	79
ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC	299
M D Y Q V S S P I Y D I N Y Y T S E P C	99
CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTG	359
Q K I N V K Q I A A R L L P P L Y S L V	119
TTCATCTTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATAAACTGCAAAGG	419
F I F G F V G N M L V I L I L I N C K R	139
CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCCTT	479
L K S M T D I Y L L N L A I S D L F F L	159
CTTACTGTCCCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG	539
L T V P F W A H Y A A A Q W D F G N T M	179
TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATC	599
C Q L L T G L Y F I G F F S G I F F I I	199
CTCCTGACAATCGATAGGTACCTGGCTGTGCTCCATGCTGTGTTTGCTTTAAAAGCCAGG	659
L L T I D R Y L A V V H A V F A L K A R	219
ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGCGTCT	719
T V T F G V V T S V I T W V V A V F A S	239
CTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCT	779
L P G I I F T R S Q K E G L H Y T C S S	259
CATTTTCCATACA	
H F P Y	

GAATTCCCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
	19
TTCCCTTCACTACAAAACCTTCATTGCTTGGCCAAAAAGAGAGTTAATTCAATGTAGACAT	119
	39
CTATGTAGGCAATTAAAAACCTATTGATGTATAAAACAGTTTGCATTTCATGGAGGGCAAC	179
	59
TAAATACATTCTAGGACTTTATAAAAGATCACTTTTTATTATGACAGGGTGGAAACAAG	239
	79
ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC	299
M D Y Q V S S P I Y D I N Y Y T S E P C	99
CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTG	359
Q K I N V K Q I A A R L L P P L Y S L V	119
TTCATCTTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATAAACTGCAAAGG	419
F I F G F V G N M L V I L I L I N C K R	139
CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCCTT	479
L K S M T D I Y L L N L A I S D L F F L	159
CTTACTGTCCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG	539
L T V P F W A H Y A A A Q W D F G N T M	179
TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATC	599
C Q L L T G L Y F I G F F S G I F F I I	199
CTCCTGACAATCGATAGGTACCTGGCTGTCGTCCATGCTGTGTTTGCTTTAAAAGCCAGG	659
L L T I D R Y L A V V H A V F A L K A R	219
ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGCGTCT	719
T V T F G V V T S V I T W V V A V F A S	239
CTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCT	779
L P G I I F T R S Q K E G L H Y T C S S	259
CATTTTCCATACAGTCAGTATCAATTCTGGAAGAATTTCCAGACATTAAAGATAGTCATC	839
H F P Y S Q Y Q F W K N F Q T L K I V I	279

SEQ ID NO.2 FIG.1b

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TTGGGGCTGGTCCCTGCCGCTGCTTGTTCATGGTCATCTGCTACTCGGGAATCCTAAAACT	899
L G L V L P L L V M V I C Y S G I L K T	299
CTGCTTCGGTGTTCGAAATGAGAAGAAGAGGCACAGGGCTGTGAGGCTTATCTTCACCATC	959
L L R C R N E K K R H R A V R L I F T I	319
ATGATTGTTTATTTTCTCTTCTGGGCTCCCTACAACATTGTCCTTCTCCTGAACACCTTC	1019
M I V Y F L F W A P Y N I V L L L N T F	339
CAGGAATTCTTTGGCCTGAATAATTGCAGTAGCTCTAACAGGTTGGACCAAGCTATGCAG	1079
Q E F F G L N N C S S S N R L D Q A M Q	359
GTGACAGAGACTCTTGGGATGACGCACTGCTGCATCAACCCCATCATCTATGCCTTTGTC	1139
V T E T L G M T H C C I N P I I Y A F V	379
GGGGAGAAGTTCAGAAACTACCTCTTAGTCTTCTTCCAAAAGCACATTGCCAAACGCTTC	1199
G E K F R N Y L L V F F Q K H I A K R F	399
TGCAAATGCTGTTCTATTTTCCAGCAAGAGGCTCCCGAGCGAGCAAGCTCAGTTTACACC	1259
C K C C S I F Q Q E A P E R A S S V Y T	419
CGATCCACTGGGGAGCAGGAAATATCTGTGGGCTTGTGACACGGACTCAAGTGGGCTGGT	1319
R S T G E Q E I S V G L *	439
GACCCAGTCAGAGTTGTGCACATGGCTTAGTTTTTCATACACAGCCTGGGCTGGGGGTNGG	1379
	459
TTGGNNGAGGTCTTTTTTAAAAGGAAGTTACTGTTATAGAGGGTCTAAGATTCATCCATT	1439
	479
TATTTGGCATCTGTTTAAAGTAGATTAGATCCGAATTC	

SEQ ID NO.2 (SUITE)FIG.1c



GAATTCCCCCAACAGAGCCAAGCTCTCCATCTAGTGGACAGGGAAGCTAGCAGCAAACC	59
	19
TTCCCTTCACTACAAAACCTTCATTGCTTGGCCAAAAAGAGAGTTAATTCRAATGTAGACAT	119
	39
CTATGTAGGCAATTAAAAACCTATTGATGTATAAAACAGTTTGCAATTCATGGAGGGCAAC	179
	59
TAAATACATTCTAGGACTTTATAAAAGATCACTTTTTATTATGCACAGGGTGGAAACAAG	239
	79
ATGGATTATCAAGTGTCAAGTCCAATCTATGACATCAATTATTATACATCGGAGCCCTGC	299
M D Y Q V S S P I Y D I N Y Y T S E P C	99
CAAAAAATCAATGTGAAGCAAATCGCAGCCCGCCTCCTGCCTCCGCTCTACTCACTGGTG	359
Q K I N V K Q I A A R L L P P L Y S E V	119
TTCATCTTTGGTTTTGTGGGCAACATGCTGGTCATCCTCATCCTGATAAACTGCAAAAGG	419
F I F G F V G N M L V I L I L I N C K R	139
CTGAAGAGCATGACTGACATCTACCTGCTCAACCTGGCCATCTCTGACCTGTTTTTCCTT	479
L K S M T D I Y L L N L A I S D L F F L	159
CTTACTGTCCCTTCTGGGCTCACTATGCTGCCGCCAGTGGGACTTTGGAAATACAATG	539
L T V P F W A H Y A A A Q W D F G N T M	179
TGTCAACTCTTGACAGGGCTCTATTTTATAGGCTTCTTCTCTGGAATCTTCTTCATCATC	599
C Q L L T G L Y F I G F F S G I F F I I	199
CTCCTGACAATCGATAGGTACCTGGCTGTGCTCCATGCTGTGTTTGCTTTAAAGCCAGG	659
L L T I D R Y L A V V H A V F A L K A R	219
ACGGTCACCTTTGGGGTGGTGACAAGTGTGATCACTTGGGTGGTGGCTGTGTTTGCGTCT	719
T V T F G V V T S V I T W V V A V F A S	239
CTCCCAGGAATCATCTTTACCAGATCTCAAAAAGAAGGTCTTCATTACACCTGCAGCTCT	779
L P G I I F T R S Q K E G L H Y T C S S	259
CATTTTCCATACATTAAAGATAGTCATCTTGGGGCTGGTCCTGCCGCTGCTTGTCATGGT	839
H F P Y I K D S H L G A G P A A A C H G	279

SEQ ID NO.3FIG.1d

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CATCTGCTACTCGGGAATCCTAAAACTCTGCTTCGGTGTCGAAATGAGAACAAGAGGCA	899
H L L L G N P K N S A S V S K *	299
CAGGGCTGTGAGGCTTATCTTCACCATCATGATTGTTTATTTTCTCTTCGGGCTCCCTA	959
	319
CAACATTGTCTTCTCCTGAACACCTTCCAGGAATTCTTTGGCCTGAATAATTGCAGTAG	1019
	339
CTCTAACAGGTTGGACCAAGCTATGCAGGTGACAGAGACTCTTGGGATGACGCACTGCTG	1079
	359
CATCAACCCCATCATCTATGCCTTTGTGCGGGGAGAAGTTCAGAACTACCTCTTAGTCTT	1139
	379
CTTCCAAAAGCACATTGCCAAACGCTTCTGCAAATGCTGTTCTATTTTCCAGCAAGAGGC	1199
	399
TCCCGAGCGAGCAAGCTCAGTTTACACCCGATCCACTGGGGAGCAGGAAATATCTGTGGG	1259
	419
CTTGTGACACGGACTCAAGTGGGCTGGTGACCCAGTCAGAGTTGTGCACATGGCTTAGTT	1319
	439
TTCATACACAGCCTGGGCTGGGGGTNGGTTGGNNGAGGTCTTTTTTAAAAGGAAGTTACT	1379
	459
GTTATAGAGGGTCTAAGATTCATCCATTTATTTGGCATCTGTTTAAAGTAGATTAGATCC	1439
	479
GAATTC	

SEQ ID NO.3 (SUITE)FIG.1e



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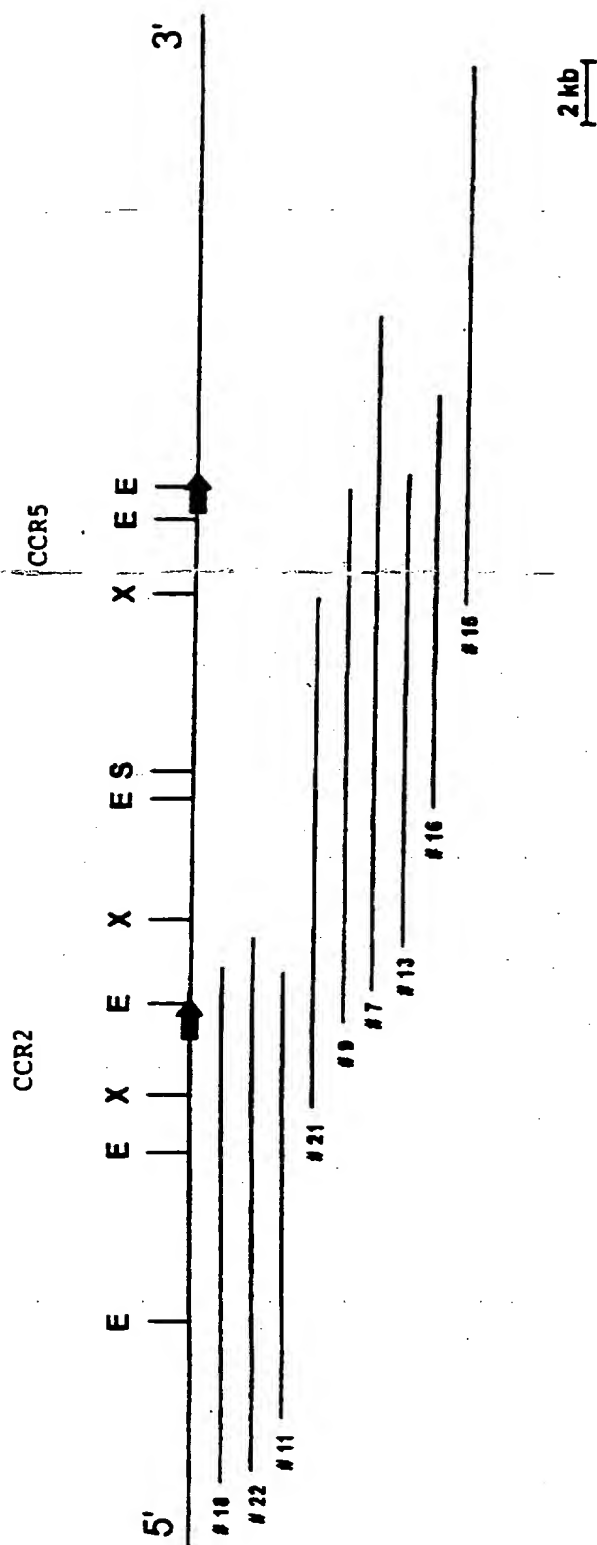


FIG. 3

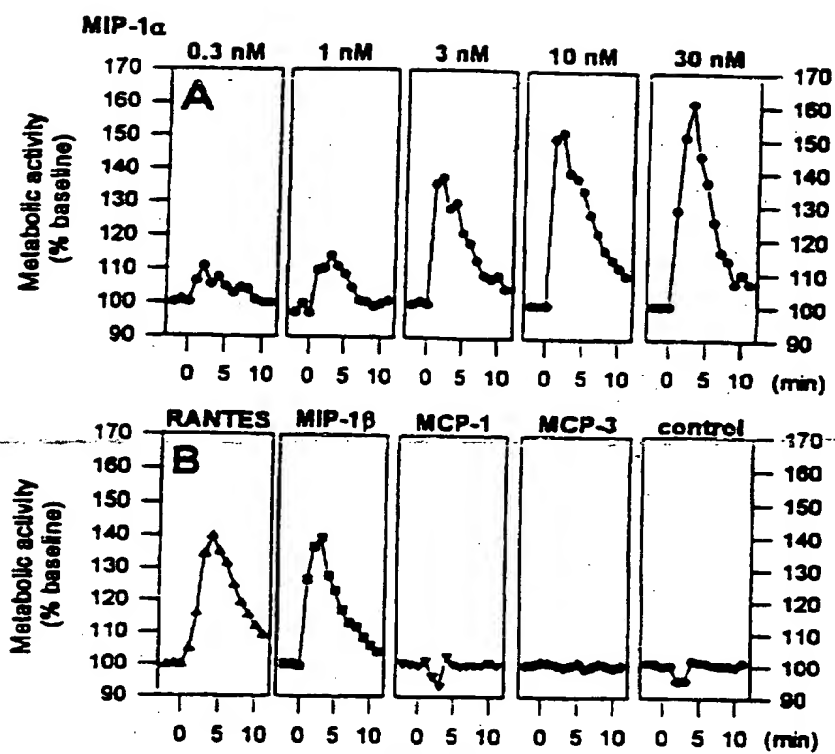


FIG. 4a

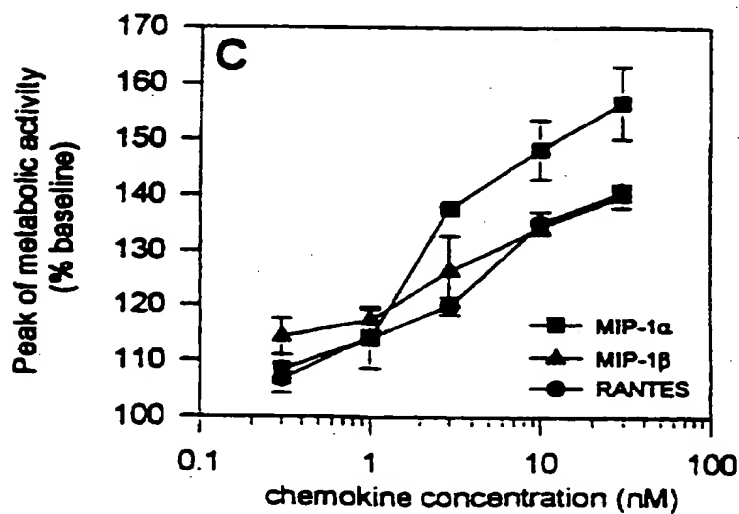
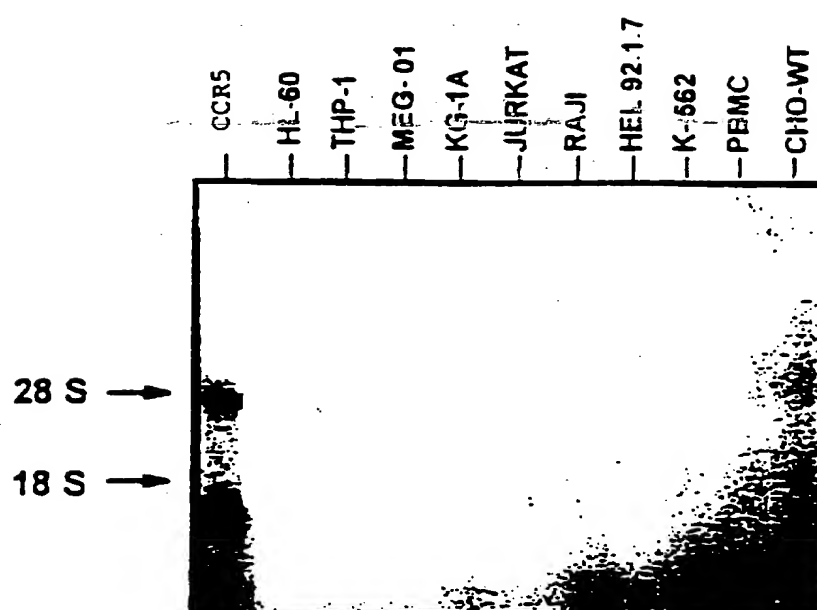
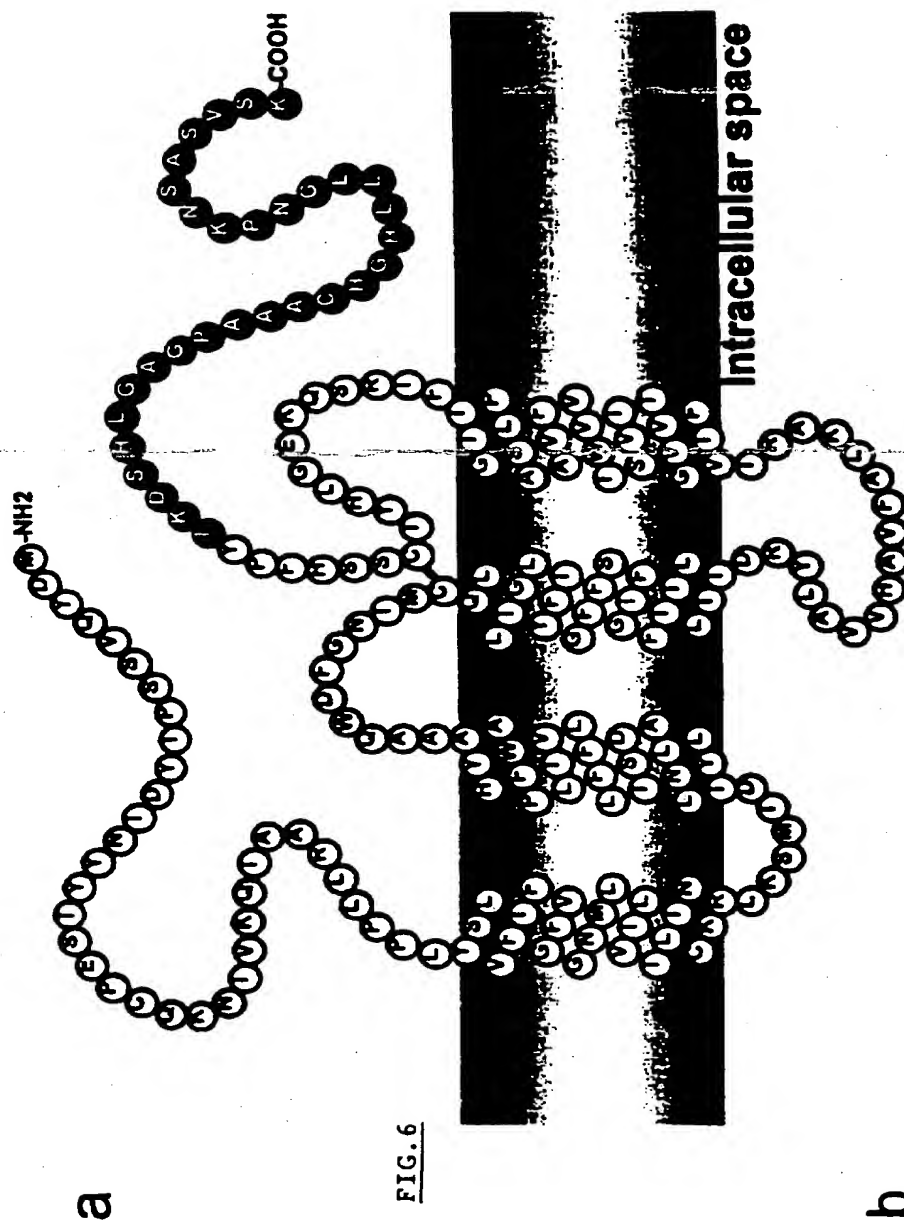


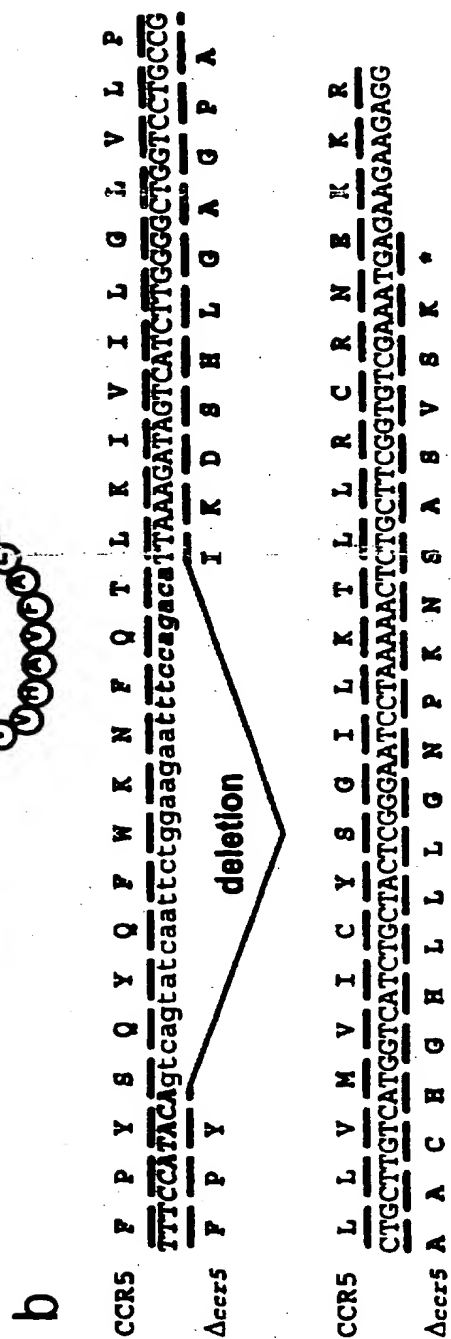
FIG. 4b

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**FIG. 5**



**FIG. 6**



A.

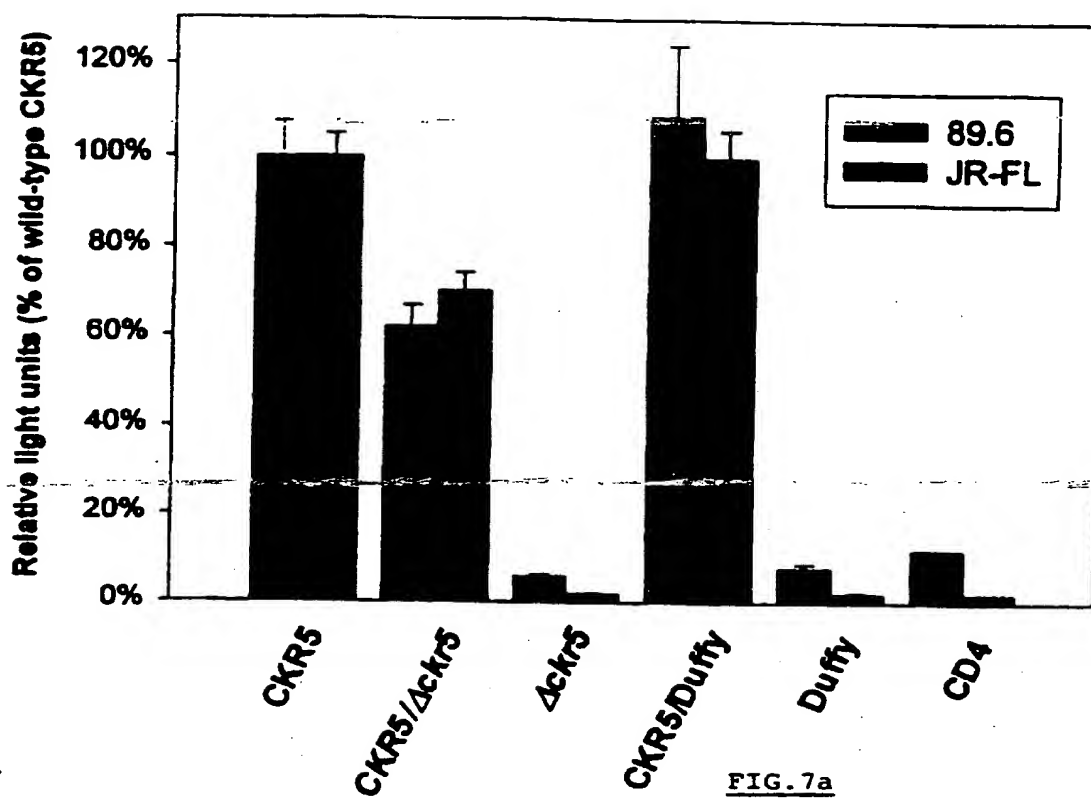


FIG. 7a

B.

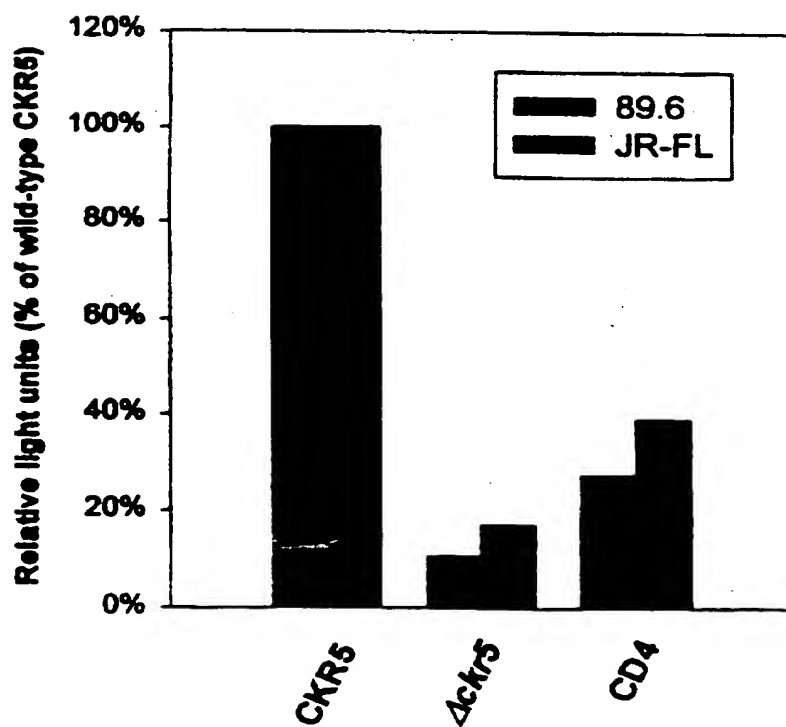
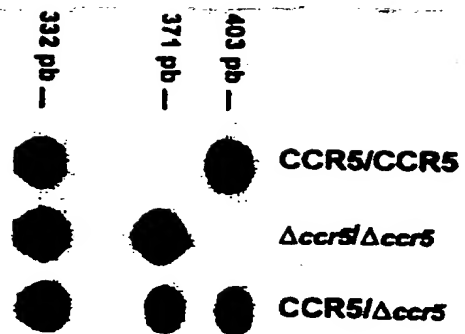


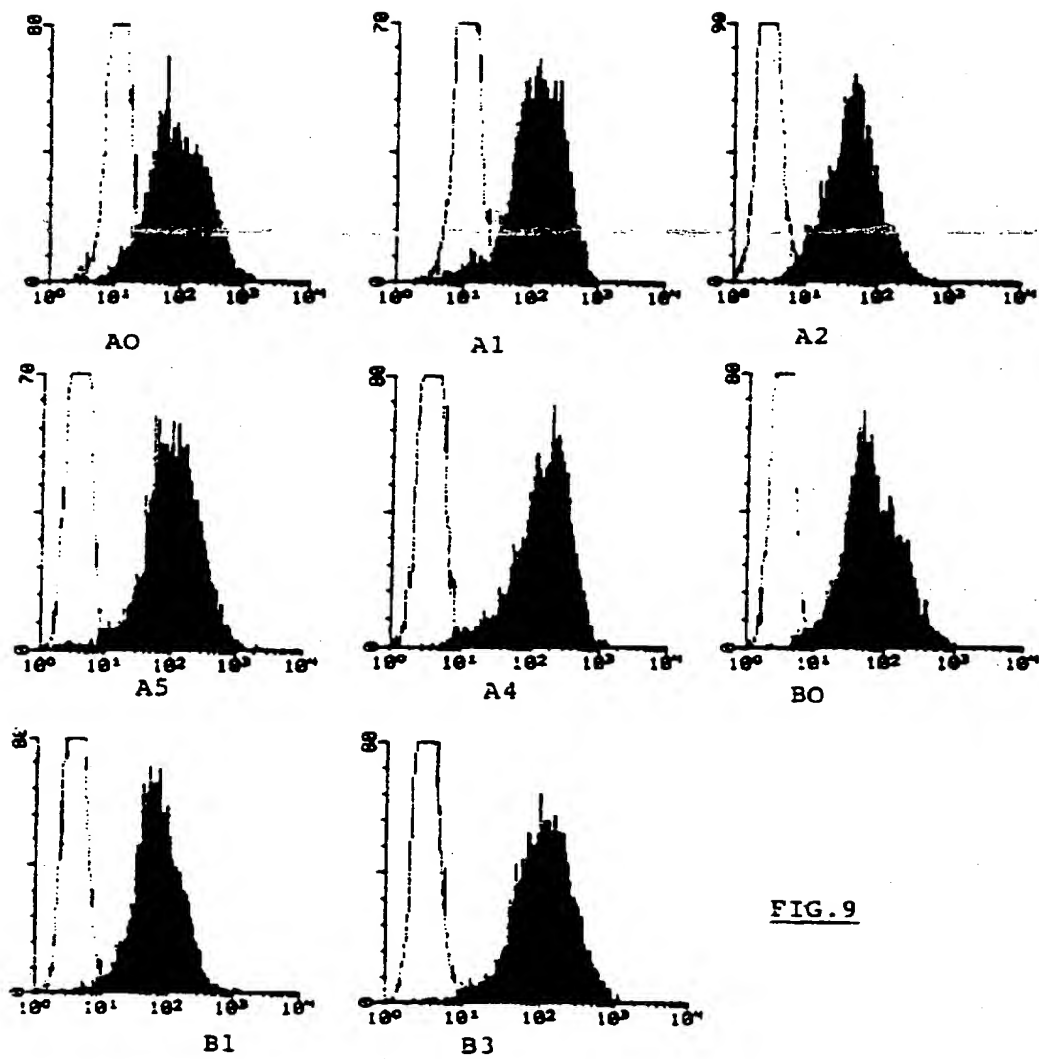
FIG. 7b



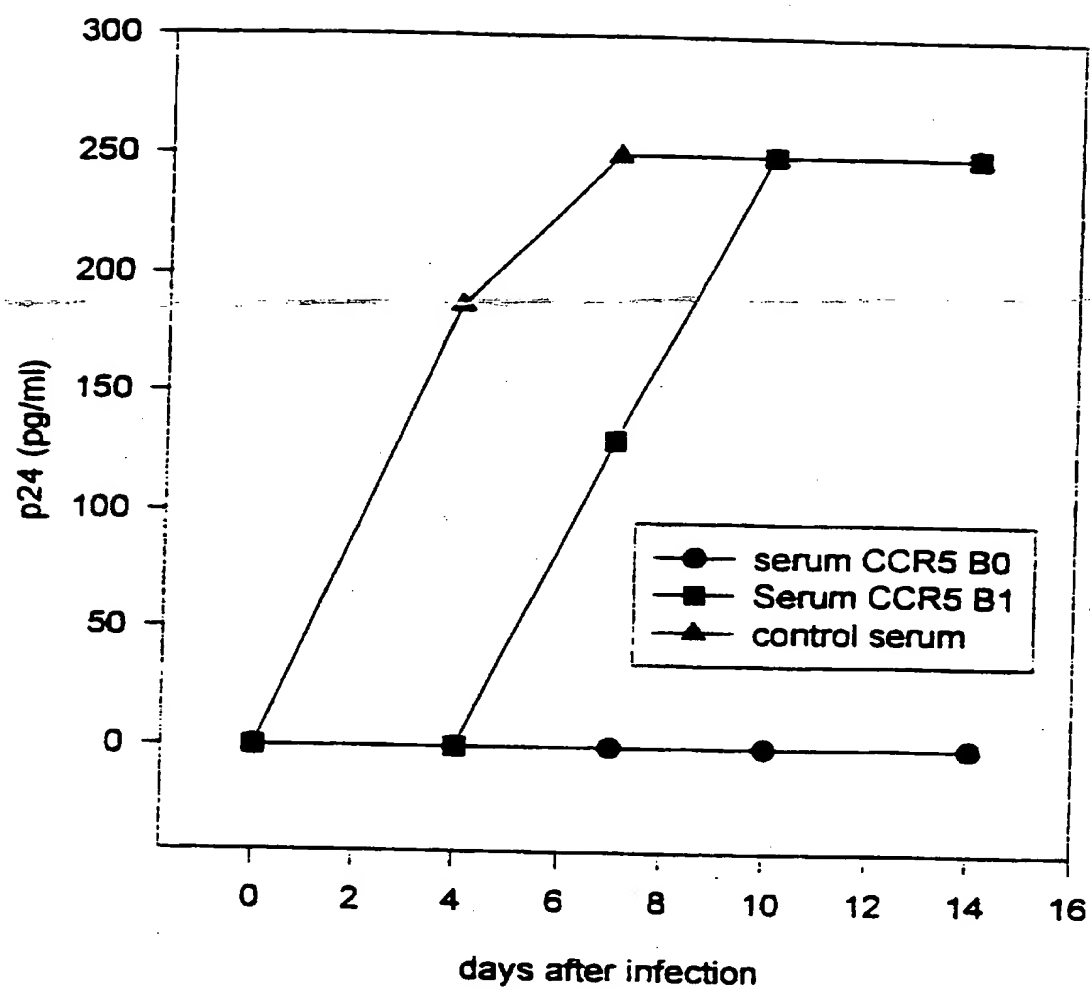
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FIG. 8

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FIG. 10

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™  
LMBP-COLLECTION**

Page 1 of Form BCCM™/LMBP/BP/4/ 97-11      Receipt in the case of an original deposit

**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for  
the Purposes of Patent Procedure**

**Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the  
International Depositary Authority BCCM™/LMBP identified at the bottom of next page**

***International Form BCCM™/LMBP/BP/4/97-11***

**To :      Name of the depositor : EUROSCREEN s.a.**

**Address : 808 route de Lennik, 8-1070 Bruxelles**

**I.      Identification of the microorganism:**

**I.1      Identification reference given by the depositor:**

**CHO-K1-pEFIN hCCR5-1/16**

**I.2      Accession number given by the International Depositary Authority:**

**LMBP1657CB**

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™**

**LMBP-COLLECTION**

Page 2 of Form BCCM™/LMBP/BP/4/97-11

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**II. Scientific description and/or proposed taxonomic designation**

The microorganism identified under I above was accompanied by:

(mark with a cross the applicable box(es)):

- ☒ a scientific description  
☐ a proposed taxonomic designation


**III. Receipt and acceptance**

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) : February 27, 1997

**IV. International Depositary Authority**

Belgian Coordinated Collections of Microorganisms (BCCM™)  
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)  
Universiteit Gent  
K.L. Ledeganckstraat 35  
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Date : June 04, 1997

M.C. Martine Vanhoucke  
LMBP Assistant Manager

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™**  
**LMBP-COLLECTION**

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**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for  
the Purposes of Patent Procedure**Viability statement issued pursuant to Rule 10.2 by the International Depositary  
Authority BCCM™/LMBP identified on the following page*International Form BCCM™/LMBP/BP/9/97-11*

To : Party to whom the viability statement is issued:

Name : Pierre Nokin  
Administrateur DéléguéAddress : EUROSCREEN s.a.  
808 route de Lennik, B-1070 Bruxelles

## I. Depositor:

I.1 Name : EUROSCREEN s.a.

I.2 Address : 808 route de Lennik, B-1070 Bruxelles

## II. Identification of the microorganism:

II.1 Accession number given by the International Depositary Authority:

LMBP1657CB

II.2 Date of the original deposit (or where a new deposit or a transfer has been  
made, the most recent relevant date) : February 27, 1997

## III. Viability statement.

The viability of the microorganism identified under II above was tested on

: April 08, 1997

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent  
viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable☐ no longer viable

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™  
LMBP-COLLECTION**

Page 2 of Form BCCM™/LMBP/BP/9/97-11      Viability statement

---

**IV.      Conditions under which the viability test has been performed:**

(Fill in if the information has been requested and if the results of the test were negative).

**V.      International Depository Authority**

**Belgian Coordinated Collections of Microorganisms (BCCM™)  
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)  
Universiteit Gent  
K.L. Ledeganckstraat 35  
B-9000 Gent, Belgium**

**Signature(s) of person(s) having the power to represent the International Depository  
Authority or of authorized official(s):**

**Date      : June 04, 1997**

  
**Lic. Martine Vanhoucke  
LMBP assistant manager**

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™  
LMBP-COLLECTION**

Page 1 of Form BCCM™/LMBP/BP/4/ 97-12 Receipt in the case of an original deposit

## Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

**Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the International Depository Authority BCCM™/LMBP identified at the bottom of next page**

**International Form BCCM™/LMBP/BP/4/97-12**

**To : Name of the depositor : EUROSCREEN s.a.**

**Address** : 808 route de Lennik, B-1070 Bruxelles

**1. Identification of the microorganism:**

**1.1 Identification reference given by the depositor:**

## AchCCR5 - SAB1A7

**1.2 Accession number given by the International Depository Authority:**

**LMBP1658CB**



**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™  
LMBP-COLLECTION**

Page 2 of Form BCCM™/LMBP/BP/4/97-12

Receipt in the case of an original deposit

**II. Scientific description and/or proposed taxonomic designation**

The microorganism identified under I above was accompanied by:

(mark with a cross the applicable box(es)):

- ☒ a scientific description  
☐ a proposed taxonomic designation

**III. Receipt and acceptance**

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) : February 27, 1997

**IV. International Depositary Authority**

Belgian Coordinated Collections of Microorganisms (BCCM™)  
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)  
Universiteit Gent  
K.L. Ledeganckstraat 35  
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):



Date : June 04, 1997

Lic. Martine Vanhoucke  
LMBP Assistant Manager

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™**  
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**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for  
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Authority BCCM™/LMBP identified on the following page*International Form BCCM™/LMBP/BP/9/97-12***To :** Party to whom the viability statement is issued:**Name** : Pierre Nokin  
Administrateur Délégué**Address** : EUROSCREEN s.a.  
808 route de Lennik, B-1070 Bruxelles**I. Depositor:****I.1 Name** : EUROSCREEN s.a.**I.2 Address** : 808 route de Lennik, B-1070 Bruxelles**II. Identification of the microorganism:****II.1 Accession number given by the International Depositary Authority:**

LMBP1658CB

**II.2 Date of the original deposit (or where a new deposit or a transfer has been  
made, the most recent relevant date)** : February 27, 1997**III. Viability statement.**

The viability of the microorganism identified under II above was tested on

: May 23, 1997

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent  
viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ viable☐ no longer viable

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™  
LMBP-COLLECTION**

Page 2 of Form BCCM™/LMBP/BP/9/97-12      Viability statement

---


**IV.      Conditions under which the viability test has been performed:**

(Fill in if the information has been requested and if the results of the test were negative).

**V.      International Depository Authority**

Belgian Coordinated Collections of Microorganisms (BCCM™)  
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)  
Universiteit Gent  
K.L. Ledeganckstraat 35  
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depository  
Authority or of authorized official(s):

  
Lic. Martine Vanhoucke  
LMBP assistant manager

Date    June 04, 1997

Référence du dossier du déposant ou du mandataire	P. RE.02/WO	Demande internationale n°	PCT BE 97/00023
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# INDICATIONS RELATIVES A UN MICRO-ORGANISME

(règle 13bis du PCT)

REC'D 17 MAR 1997

DEPOSE  
WFO

PCT

A. Les indications ont trait au micro-organisme visé dans la description page <u>17</u> ligne <u>23</u>	
B. IDENTIFICATION DU DEPOT <span style="float: right;">D'autres dépôts font l'objet d'une feuille supplémentaire <input checked="" type="checkbox"/></span>	
Nom de l'institution de dépôt BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS (BCCM) LABORATORIUM VOOR MOLECULAIRE BIOLOGIE - PLASMIDENCOLLECTIE (LMBP)	
Adresse de l'institution de dépôt (y compris le code postal et le pays) Universiteit Gent K.L. Ledeganckstraat 35 B-9000 GENT BELGIUM	
Date du dépôt	n° d'ordre
C. INDICATIONS SUPPLEMENTAIRES (le cas échéant) <span style="float: right;">Une feuille supplémentaire est jointe pour la suite de ces renseignements <input type="checkbox"/></span>	
D. ETATS DESIGNES POUR LESQUELS LES INDICATIONS SONT DONNEES (si les indications ne sont pas données pour tous les Etats désignés)	
E. INDICATIONS FOURNIES SEPAREMENT (le cas échéant) Les indications énumérées ci-après seront fournies ultérieurement au Bureau international (spécifier la nature générale des indications p. ex., "n° d'ordre du dépôt")	

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<p>Réservé au Bureau international</p> <p><input type="checkbox"/> Cette feuille est parvenue au Bureau international le :</p> <p>Fonctionnaire autorisé</p>
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